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Evaluation of Genomic Instability in the Abnormal Prostate

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14. ABSTRACT
The aim of this study is to investigate the field effect in prostate cancer, the relationship between tumor and nearby histologically normal tissues compared to truly disease free prostate tissue. Identification of changes within tumor adjacent tissues has two possible clinical implications: prognosis and diagnosis. Several tools are being used to investigate this effect, specifically the assessment of telomere length, allelic imbalance, and methylation status, all markers of genomic instability. Microarray studies will be used to aid in the identification of additional gene expression changes occurring between tumor and histologically normal tissues compared to truly disease free tissue. While telomere length and allelic imbalance have been shown to correlate with outcome, it is expected that, when compared with truly normal tissue from disease-free prostates, several progressive changes will be seen, as has been found in prostate cancer cell lines. The proposed study will allow for interaction with other scientists, exposure to new technologies, teaching and continued patient interaction, all of which are important to the physician scientist.

15. SUBJECT TERMS
Prostate Cancer, Telomeres, Methylation, Micro array, Slot Blot, Allelic Imbalance

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I. Introduction

The aim of this study is to investigate the relationship between tumor and tumor adjacent histologically normal (TAHN) prostate tissues, also referred to as the field effect. When compared with truly disease free prostate tissue, identification of changes within tumor adjacent tissues has two possible clinical implications: prognosis and diagnosis. Several tools are being used to investigate the field effect, specifically the assessment of telomere length, allelic imbalance, and methylation status, all markers of genomic instability. Microarray studies will be used to aid in the identification of additional gene expression changes occurring between tumor and histologically normal tissues compared to truly disease free tissue. While telomere length and allelic imbalance of tumor tissue have been shown to correlate with staging, it is expected that, when compared with truly normal tissue from disease-free prostates, changes will be seen in the nearby histologically normal tissues as well. The proposed study will allow for interaction with other scientists, exposure to new technologies, teaching and continued patient interaction, all of which are important to the physician scientist.

Hypothesis and Rationale

It is currently thought that a multi-step process is involved in the development of prostate cancer. Preliminary data from our laboratory suggest that telomere content (TC) and allelic imbalance (AI) are altered in both tumor and tumor adjacent tissue, and that these changes precede histologic changes. It is reasonable to extrapolate from this that normal appearing tissue may have diagnostic properties. Our data also suggest that there is a relationship between the level of genomic instability and prostate cancer relapse, indicating that this tissue may also have prognostic significance. Because the TC and AI modalities have previously shown correlation between staging and outcome, these will be used to determine the effectiveness of a PCR-based promoter methylation assay. Additionally, this study will incorporate microarray analysis to help guide the determination of where to look for changes in methylation status based on expression changes, as this will allow determination of expression differences between tumor, tumor adjacent tissues, and disease free prostate tissues.

Specific Aim #1: *Further assess and refine the use of the allelic imbalance assay in predicting potential disease relapse in retrospective and prospective studies of prostate cancer.*

Specific Aim #2: *Compare methylation states of genes known to be associated with prostate cancer, such as GSTP1, P504S, and CD44, between tumor cells, TAHN tissue and normal prostate tissue from men without cancer.*

Specific Aim #3: *Assess with microarray technology characteristic changes in gene expression relevant to prognosis in prostate cancer, and determine if this profile extends to surrounding histologically normal cells.*

II. Body

IIa. Materials and Methods

Patient Specimens:

Samples for use in all arms of the study have been identified and collected from the New Mexico Tumor Registry (NMTR), National Cancer Institute Cooperative Human Tissue Network (CHTN, Nashville, TN), Cooperative Prostate Cancer Tissue Resource (Pittsburg, PA) and the University of New Mexico School of Medicine (UNMSOM). Control RNA, consisting of 9 pooled patients (from sudden death cases), was obtained from Ambion (Austin, TX) for use as the control in the microarray study.

Cell Lines:

DNA from cell lines (LnCaP, DU146, C4-2b, and PC-3) were used to determine if the primers and sodium bisulfite treatment were functioning properly. These lines represent a range of prostate cancer and gene promoter methylation.

Methylated DNA Control:

Universally methylated DNA (CpGenome™ Universal Methylated DNA) was obtained from Millipore (Temecula, CA) for use as a positive control in the methylation studies.

DNA Isolation:

All samples were fresh-frozen tissue with the exception of those from the CPCTR, from which DNA was isolated using a commercially available kit (DNeasy, Qiagen, Valencia, CA). The tissues from CPCTR were formalin-fixed, paraffin embedded sections, which were first deparaffinized and rehydrated prior to removal of the tissue from the slides, followed by DNA extraction in the same manner as the other samples. Following DNA isolation and in preparation for the Telomere Content (TC) assay, the dsDNA concentration was measured using PicoGreen (Quant-iT™ Picogreen ® dsDNA Kit, Molecular Probes, Eugene, OR) according to the manufacturer's protocol. For the Allelic Imbalance (AI) assay and methylation studies, DNA was quantified using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Wilmington, DE).

RNA Isolation and Labeling:

RNA was isolated with commercially available kits (Qiashredder and RNeasy kits, Qiagen, Valencia, CA) from the set of 12 cases identified for use in the microarray study. RNA was assessed by NanoDrop™ (Thermo Scientific, Wilmington, DE) and the Agilent Bioanalyzer 2100 (Agilent, Foster City, CA) to determine quantity and quality of the RNA isolated.

RNA from 6 matched cases (*i.e.* a TAHN and Tumor sample from the same ~~one~~ patient) was then reverse transcribed into complementary DNA (cDNA) using the Retroscript™ RT Kit (Ambion, Austin, TX), followed by labeling with either Cy3 (pooled control RNA) or Cy5 (either tumor or TAHN pool) fluorescent cyanine dyes. Labeling was achieved by synthesizing the cDNAs in the presence of amino allyl dUTP (Sigma-Aldrich, St. Louis, MO) followed by chemically coupling of either Cy3 or Cy5 monofunctional dye (Amersham-Pharmacia Biotech, Arlington Heights, IL) to the cDNA. This process avoids biased incorporation of the dyes during reverse transcription. Dye incorporation was measured by NanoDrop™ (Thermo Scientific, Wilmington, DE) on an individual sample basis prior to pooling of the experimental groups in order to ensure equal representation of each sample in the pool.

Telomere Content (TC) Assay:

Assessment of TC was carried out as described previously (1, 2). Briefly, this is a blot assay that allows for chemiluminescent detection and quantitation of telomere content using a labeled telomere-specific probe.

Allelic Imbalance Assay:

Assessment of AI was performed as previously described (9, 11, 12) using the AmpFlSTR® kit (Applied Biosystems, Foster City, CA) which contains reagents that amplify 16 different short tandem repeat (i.e. microsatellite) loci within a single multiplex reaction.

Microarray Expression Analysis:

Glass-slide-spotted-expression microarrays of the Qiagen Human Genome Oligo Set Version 3.0 (Qiagen) were used for this investigation. The arrays contained 37,123 transcripts, including 24,650 known genes, the rest being expressed sequence tags (ESTs) and controls. The design of these arrays is based on the Ensembl Human 13.31 Database (http://www.ensembl.org/) and on the Human Genome Sequencing Project. Equal parts of Cy3 and Cy5 labeled cDNAs were then combined and competitively hybridized to the microarray slides using the GeneTAC Genomic Solutions machine and protocol (Genomic Solutions Inc, Ann Arbor, MI). Following hybridization and washing, the slides were scanned at 532nm and 635nm using the Axon 4000A scanner (Axon Instruments, Union City, CA), and the signal data was processed using Axon GenePix Pro 5 software (Axon Instruments). Fluorescence intensities of the Cy3 and Cy5 dyes were determined for each oligonucleotide spot, followed by visual inspection prior to importing into Acuity 3.0 (Molecular Devices, Sunnyvale, CA). This program was utilized to normalize the data and allow for comparison between the replicates using standard quality calls (background removal, linear regression ratio >0.6, signal to noise ratio >3.0). Only data passing these quality filters were utilized in the present analysis. Sample groups, i.e. tumor and TAHN pools, were run in triplicate hybridizations.

Quantitative (real time) Reverse Transcriptase PCR:

Quantitative Real Time PCR (qRT-PCR) was used to verify the results of the microarray expression analyses. Samples from both the microarray (MA) and the independent validation array (VA) sets were individually analyzed in quadruplicate for each selected gene/primer set. Approximately 1 µg of RNA from the samples was converted to cDNA using the Retroscript™ RT Kit (Ambion) according to the manufacturer's protocol using random decamers. The cDNAs were subsequently diluted 1:5 for use in the PCR reactions.

The gene evaluated for mRNA expression to date is early growth response protein 1 (EGR-1). The sequence for PCR primers was previously published (3). mRNA levels were quantitated using the Sybr Green real-time PCR assay kit (Applied Biosystems, Foster City, CA) in a 25uL reaction, using 0.5uL of the diluted cDNA. Primers were used at a final concentration of 1 µmol for the forward and 1.5 µmol for the reverse in the PCR reaction. PCR reactions were carried out under the following cycling parameters: 95˚C for 10 minutes followed by 40 cycles of 95˚C for 15 seconds, and 60˚C for one minute using the Gene Amp® 7000 Sequence Detection System (Applied Biosystems). Baseline fluorescence was determined during cycles 6-15.

The levels of EGR-1 were determined using the ΔΔCt method, where the threshold of detection of the genes of interest were compared to the house keeping gene TATA binding protein (TBP). This method was chosen because the amplification efficiency of their primers was determined to be similar to that of the control transcript.

Sodium Bisulfite Treatment and Quantitative Methylation Specific PCR (Q-MSP):

Following DNA extraction, DNA was treated with the commercially available sodium bisulfite-based kit CpGenome fast DNA modification kit (Millipore, Temecula, CA) to cause deamination of unmethylated cytosines in the CpG repeats.

Primers used here were previously published (4-6) (Table 1). Semi-quantitative methylation specific PCR (QMSP) utilized methylated DNA specific TaqMan probes to detect methylated samples. In this technique, 1 uL of the sodium bisulfite treated DNA was combined with 10 uL of 2x TaqMan Universal PCR Kit (Applied Biosystems, Foster City, CA), 600 nmol of each primer, 200 nmol probe, and the remaining volume water for a total of 20 uL. The

reactions were run on an ABI PRISM 7000 real time PCR machine with the following protocol: Initial denaturation at 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds then 60°C for one minute (5, 7, 8). All samples were run in quadruplicate, and a promoter specific to unmethylated β-actin was used as the internal control. To determine levels of methylation, the delta Ct of a sample was divided by the delta Ct of β-actin and then multiplied by 100 to give a representative methylation level. Controls included a control reaction without template and a fully methylated DNA control (CpGenome™ Universal Methylated DNA, Millipore, Temecula, CA). In order for the data to be considered acceptable, there needed to be at least three interpretable results, which were then averaged and used in the calculation of relative methylation. Primers were designed to generate products between 80-200bp in size.

IIb. Results

Telomere Content Study

To date, results of the TC studies are consistent with those previously published by this laboratory (9, 10). Specifically, TC in TAHN tissues is more similar to TC in tumor tissues than normal tissues, indicating the presence of a field of abnormal cells surrounding the tumor. However, the purpose of the current study is to verify these findings in an additional study cohort. Prospective samples are being collected, however the time frame of the study will not allow for follow up information to be collected. Instead a retrospective cohort (CPCTR) has been identified and is currently being analyzed.

Allelic Imbalance Study

AI has been assessed as previously using an assay previously described in the archival tissues. The findings indicate that TAHN tissue is abnormal when compared to truly disease-free tissue. Again, as mentioned in the previous section regarding TC, the purpose of this study was to verify previous findings demonstrating field effect and potential use as a diagnostic tool. Prospective samples are being collected, however the time frame of the study will not allow for follow up information to be collected. Instead a retrospective cohort (CPCTR) has been identified and is currently being analyzed.

Microarray expression analysis.

RNA expression levels are reported here as ratios of Cy3/Cy5 signals for individual transcripts, where the Cy3 and Cy5 fluorescent cyanine dyes were used to label cDNA from experimental (tumor or TAHN) and pooled cancer-free control tissues, respectively. While a ratio of 1.0 would thus indicate no change in expression compared to cancer-free controls, there is the possibility of dye bias due to differential incorporation of Cy3 and Cy5 during cDNA synthesis, or due to differential hybridization of Cy3- and Cy5-labeled cDNAs to target probes. To estimate the extent of potential dye bias, we labeled paired aliquots of control cDNA from cancer-free prostatic tissues with Cy3 and Cy5, combined equal amounts of the preparations, and hybridized them to a microarray set. Fluorescence analysis revealed a mean Cy3/Cy5 ratio of 1.27 ± 0.35 standard deviation (SD), a median ratio of 1.22, and a coefficient of variation of 27.3% for all transcripts (Table 3). In contrast, the means ± SD and coefficients of variation determined for the TAHN and tumor experimental sets were 1.58 ± 0.61 and 38.6%, and 1.63 ± 0.75 and 46.1%, respectively. Statistical analysis for the distribution of values for all detected transcripts revealed significant differences ($p<0.05$) for the tumor and TAHN microarray data from the Cy3/Cy5 dye bias test (Table 3). While this result indicated a minimal dye bias for Cy3 fluorescent cyanine cDNA incorporation and/or target hybridization, we considered all transcripts in the experimental sets with an expression ratio of <1.27 as equally or under-expressed compared to normal cancer-free prostatic tissues in order to avoid false positive assignment of over-expressed genes. Consideration of the Cy3/Cy5 dye bias is important because we focused our analyses of the microarray expression experiments on over-expressed transcripts,

since over-expression of a protein marker in TAHN tissues would be amenable to positive identification and could thus be used in diagnostic tests. The results of the array found 3769 transcripts that were mutually expressed in both tumor and TAHN tissues, 1810 of which were expressed above the Cy3/Cy5 dye bias of 1.27 (see Table 2 for a partial listing).

Egr-1 expression was found to be increased in the pooled TAHN microarray set. RT-PCR was chosen to validate this result. RT-PCR analysis found Egr-1 levels to be elevated on an individual basis for the 6 TAHN microarray samples, as well as the independent TAHN validation set. Further, RT-PCR analysis revealed that Egr-1 expression was also elevated in tumor samples in both the microarray and the validation sets (Figure 6). RT-PCR analysis of eight normal samples from CHTN further revealed a slightly lower level of expression than indicated by the pooled control prostate RNA used in the microarray study (Figure 7).

Methylation Study

Gene promoter methylation was found to be highly variable between the cells lines (LnCap, PC-3, DU-145, and C4-2b) investigated (Figure 1). In all eight instances, normal DNA was found to lack methylation at the promoters of the genes of interest (Figure 2), and all normal samples were unmethylated for β-actin.

Tumor and TAHN samples showed methylation for some genes and not others (Figure 3). While the GSTP1 assay successfully identified methylation in cell lines, it did not detect methylation in patient samples, contrary to previously published studies of GSTP1 methylation in cancer. Troubleshooting is underway to determine why these results are inconsistent. Rar-β2 was found to be methylated in one instance of tumor DNA. APC was methylated in four tumor samples, but none of the TAHN samples (Figure 5). RassF1A was showed frequent promoter methylation in tumor samples (Figure 3), and was also found in four samples to be methylated in the matched TAHN tissues (Figure 4).

IIc. Discussion

Telomere Content Study

Because telomerase is frequently activated in cancer cells, it follows that telomere length of these cells will be different from normal, healthy cells, and this has indeed been observed to be the case. Our laboratory has developed an assay to measure telomere content (TC), a surrogate for telomere length, to evaluate telomeres of cancer and normal cells (1, 2). Additionally, our laboratory has used the TC assay to investigate field effect in the tumor adjacent histologically normal tissues of breast and prostate cancer. These studies have established that the telomeres of tumor cells are abnormal compared to disease-free cells, that shorter telomeres are associated with a poorer outcome, and that a field of abnormal cells surrounds a tumor (2, 9, 10, 13, 14).

Based on our preliminary studies, we propose that TC predicts disease-free survival in men with prostate cancer. To confirm and refine this finding, we conducted a retrospective study comparing TC in matched biopsy and prostatectomy tissues, in which the patients differ in recurrence outcome. This experiment will investigate whether TC can be used to predict disease in biopsy tissues. Additionally, use of the CPCTR study cohort will potentially uncover the relationship between matched biopsy tissues and prostatectomy tissues. Because the tissues are patient matched, this study will be able to determine what kind of relationship exists between the two sample types, if any.

Allelic Imbalance Study

Genomic instability is a well accepted phenomenon of cancer (15-17). Instability can be reflected in loss of heterozigosity, and so other studies have endeavored to determine if loss of heterozigosity studies can be used to detect prostate cancer (18). However, this approach assumes that the genomic changes are consistent from case to case of prostate cancer. Viewed from the perspective that the entire genome becomes unstable during carcinogenesis, the assay

employed by our laboratory is capable of capturing this picture of wide spread genomic instability as opposed to looking for a single event. Additionally, our assay avoids the requirement of a 'normal' control sample, reducing the amount of tissue or other biologic samples required from the patient. Because the assay is PCR based, only a small amount if tissue is required, such as a biopsy needle core. Previous studies from our laboratory in both breast and prostate cancers have demonstrated the presence of a field of abnormal cells surrounding the tumor as demonstrated by allelic imbalance (9). Taken together, results have shown that the histologically normal tissue from a cancerous prostate is indeed informative using this assay.

While this study originally proposed to look for prostate cancer specific microsatellite markers, it makes more sense to use an assay that is general to more than one cancer. Additionally, the commercial availability of the AmpFlSTR® kit (Applied Biosystems, Foster City, CA) makes it the type of assay that can easily move into the clinical setting. For these reasons, the study has evolved to validating the use of the AI assay for possible clinical use.

Based on our preliminary studies, we propose that AI, like TC, predicts disease-free survival in men with prostate cancer. To confirm and refine this finding, we propose to conduct a retrospective study comparing AI sensitivity and specificity in both biopsy and prostatectomy tissues to patient outcome, in which the patients differ in disease stage at time of diagnosis and recurrence outcome. These experiments will investigate whether AI can be used to predict disease recurrence regardless of stage at diagnosis. Based on preliminary studies suggesting that AI can predict disease-free survival in men with differing pathological grades, we also propose to determine if AI has utility as a marker in prostate cancer diagnosis and staging that is more sensitive than PSA.

Microarray Study

Due to a limited amount of tissue and RNA, samples from 6 matched patient cases were pooled such that there was a pool of TAHN-derived and a pool of tumor-derived cDNAs. This allowed the preservation of the same 6 patient RNAs in sufficient quantity to verify the microarray findings by RT-PCR. Additionally, there remained the second set of 6 samples which became an independent validation set for RT-PCR.

A brief list of the transcripts determined to be the most overexpressed by microarray analysis is found in Figure 6. The data generated thus far will now be verified through the use of RT-PCR to validate the findings in both the microarray set and the independent validation set. EGR-1 has already been verified by RT-PCR to be elevated in both tumor and TAHN tissues, indicating Egr-1 may be a useful diagnostic tool when biopsies are histologically normal. Elevated expression of Egr-1 is consistent with previous findings regarding Egr-1 and prostate caner (19-22). Work is continuing within our laboratory regarding this gene to determine if this is the case.

The finding of over 1800 genes over expressed and in common between the TAHN and Tumor tissues is highly indicative of a field of abnormality being present in the diseased prostate. Also of note is the use of bulk tissue to perform this study. The results of the study imply that such extreme measures as laser-capture microdissection or other means of enrichment are not necessary to detect the signature of a genetically abnormal field of cells that may be indicative of cancer. By not using such sophisticated technologies and techniques, and using bulk tissue and common technology such as RT-PCR instead, it is more likely that such a test could be used in the clinical setting.

Methylation Study

While data regarding the methylation of cell lines is included here, the cell lines themselves are only suitable for determining if the assay itself is working, as cell line methylation is variable between cell lines and over time (23). Additional samples from the CPCTR cohort were evaluated for suitability for methylation evaluation. However, due to the age and fixing processes of these samples, the DNA was either too degraded to begin with or was

degraded beyond use by the sodium bisulfite treatment. Additional sources of suitable DNA are being investigated, including the prospective study.

While global de-methylation is associated with the cancer genome, it is well known that methylation silencing of individual genes is also common. GSTP1 methylation is a well established phenomenon in cancer cells (7, 24). However, while several studies have evaluated many genes, including GSTP1, RassF1A, Rar-β2, and APC (4-8, 24-26), these studies have been plagued by a lack of proper controls, in that studies need to include truly normal, disease-free tissue, not tumor adjacent tissues, for establishing a base line level of methylation. This is particularly important in methylation studies where variable levels of methylation have been observed not only in tumors but in other pathologies of the prostate as well. This study endeavored to demonstrate why this is important by showing the existence of a field of altered cells in the tissues surrounding the tumor. As illustrated by the Rar-β2 results, this field of alteration does exist around a tumor, but as evidenced by the APC results, the extent of alterations in adjacent tissue is variable. While this study is not definitive, it does agree with published data, particularly a recent study focusing on field effect and the same four genes (27), and indicates that further investigation of these genes is warranted. Further investigation may lead to a new diagnostic tool to detect prostate cancer with out the presence of tumor cells in a biopsy, as well as differentiate between cancer and other pathologies such as PIN.

III. Key Research Accomplishments

IIIa. Research Accomplishments

- The assay for detecting methylation at specific gene promoters has been developed (table 1).
- Methylation of prostate cancer cell line models has been characterized for specific promoters. (Appendix A, figure 1).
- Methylation of normal prostate has been characterized. (Appendix A, figure 2).
- Gene promoter methylation of prostate tumor and tumor adjacent tissue in a collection of matched samples has been characterized for 3 sets of genes. (Appendix A, figure 3, 4, 5).
- Methylation within the set of cases currently characterized has shown changes between tumor and tumor adjacent tissues. (Appendix A, figures 4, 5).
- Characterization by microarray and validation of the results has been completed. (Appendix A, figures 6, 7, tables 2, 3).
- Data has been presented at several conferences.
- A paper has been co-authored by the candidate regarding field effect in breast tissue. (Appendix B).
- A paper has been co-authored by the candidate demonstrating the use of allelic imbalance as a measurement of genomic instability. (Appendix C).

IIb. Training and Educational Accomplishments

The student has had continuing opportunities to work and interact with oncologists, pathologists and other PhD scientists who specialize in prostate cancer. These interactions have occurred through tumor board meetings, journal clubs, urology rounds, special seminars and direct interaction within the clinical setting as well as the laboratory. Training in microscopy has taught the student recognition of the various pathologies of the prostate. Additionally, she has been active in patient enrollment for prognostic studies and has learned how to design a study, and the administrative requirements associated with clinical research. This type of interaction is also valuable, as it provides ongoing interaction with patients, something the student feels is important to her career as a physician scientist.

On an educational level, the student has completed all required course work for completion of a PhD degree. The student has also completed her Comprehensive Exam in September of 2007. The student has also assisted in the writing and the co-instruction of a section of the upper-level undergraduate course, Biochemical Laboratory Methods. The student has aspirations of continuing her career in research and remaining in academia and felt teaching provided an opportunity to develop the essential teaching skills need for her chosen career path as a physician scientist.

Training and Educational Milestones (3 tasks)

Task 1: -Develop ability to identify the morphology and characteristics of prostate tissue from normal to metastatic cancer. Learn the use of special stains and histological markers in prostate pathology. This will be done under the instruction of Dr. Nancy Joste, Chief of Surgical Pathology.
Months 1-12 **Completed with modification**

The student has learned identification of various stages of prostate cancer, as well as the normal pathology of the prostate gland. The training was carried out at the Veteran's Affairs Hospital (VAH) in Albuquerque in the pathology department with Dr. Massie. This modification occurred as the VAH has more prostate cases and the student has been enrolling patients here and Dr. Massie, the head of the Pathology Department, has been providing pathologic information regarding study subjects.

Task 2: -Attend clinics with oncologists (Dr. Ian Rabinowitz, Dr. Anthony Smith) at the University of New Mexico Hospital for the purpose of directly observing current detection, diagnosis, and treatment of prostate cancer, and to learn about the patient's interactions, perceptions and concerns with these current modalities. Attend oncology rounds and meetings at the Cancer Research and Treatment Center to expand general and detailed knowledge base of oncology.
Months 1-36 **Ongoing with modification**

The student has been observing surgeries at the VAH with Dr. Michael Davis. This change was made due to the higher frequency of prostatectomies at the VAH. The student has been attending urology rounds as well as directly interacting with prostate cancer patients.

Task 3: -Present ongoing work at local and national meetings.
Months 12-36 **Ongoing**

The student has presented at several conferences this past year, including the MD/PhD conference in Keystone, Colorado and IMPaCT meeting in Atlanta, Georgia.

IV. Reportable Outcomes

Abstracts

Sevilleta Annual Biochemistry Retreat, April 21, 2007, Sevilleta, NM

The Evaluation of Genomic Instability by Methylation Status in the Abnormal Prostate
Christina Haaland, Christopher Heaphy, Jeffrey Griffith, PhD

Introduction
Prostate cancer is the second most common cause of cancer related death in men after lung cancer, and the incidence of prostate cancer increases significantly with advanced age. It is currently accepted that tumorigenesis is a multi-step process where there is accumulation of genetic and epigenetic changes, altering the normal cellular regulatory mechanisms. Previously our laboratory has shown that telomere content (TC) and allelic imbalance (AI), both measures of genomic instability, correlate strongly with clinical outcome in prostate and breast cancers. More importantly, we have demonstrated that the changes observed in tumors are also present in tumor adjacent histologically normal (TAHN) tissues, indicating that genetic changes indicative of tumor progression precede histological differentiation. Further, these changes imply that markers of genetic instability in seemingly normal tissue proximal to prostate tumors also have prognostic significance.
Purpose
This leads to the question are these genetic changes in the TAHN prostate tissues limited to TC and AI, or do they include epigenetic changes as well? Evaluation of promoter methylation may allow for the evaluation of new markers for diagnostic or prognostic use in tissue obtained from needle core biopsies.
Methods
Detection of methylation status is a PCR-based assay performed through the use of specific primers designed for regions of DNA where methylation occurs. These areas are the CG repeats found mainly within the promoters of genes. Following treatment with sodium bisulfite, CG repeats without methylation, those promoters that are transciptionally functional, are chemically changed into TG repeats, allowing for selective detection.
Results
Currently we have also characterized the methylation status of the genes GSTP1, Rar-β2, APC, and RassF1A in prostate cancer model cell lines, disease free prostate tissue and patient samples of both tumor and matched TAHN tissues. β-actin was used as the unmethylated internal reference gene. Cell lines included PC-3, DU145, LnCaP and C4-2b. Four disease free samples were obtained from autopsy material of disease free prostates. Eight matched patient samples have been characterized to date. Cell lines demonstrated high levels of methylation. Disease free prostates demonstrate a lack of methylation for all genes. Methylation levels are more variable in the matched patient samples, and more samples are being characterized to evaluate potential patterns.
Future Directions
Additional samples and genes are being identified to clarify the significance of methylation in tumor and TAHN tissues of the prostate.

Medical Scientist Training Program Annual MD/PhD Student Conference, July 27-29, 2007, Keystone Colorado

The Evaluation of mRNA Expression and Methylation Status in the Abnormal Prostate
HAALAND, C.M.*, HEAPHY, C.M., GRIFFITH, J.K.

Prostate cancer is the second most common cause of cancer related death in men and the incidence increases significantly with advancing age. Tumorigenesis is a multi-step process characterized by the accumulation of genetic and epigenetic changes, thus altering the normal cellular regulatory mechanisms. However, these histological changes may be missed in a needle core biopsy of the prostate; therefore, molecular markers are needed to increase the accuracy of diagnosis.
Our laboratory has shown that telomere content (TC) and allelic imbalance (AI), both markers of genomic instability, are altered within tumor tissue and correlate strongly with clinical outcome measures in prostate and breast cancers. More importantly, we have demonstrated that these changes are also present in tumor adjacent histologically normal (TAHN) tissues located 1 cm from the tumor margin, implying that these markers exist in seemingly normal prostate tissues which may have prognostic and diagnostic significance.
We hypothesize that the changes in TAHN tissues are not limited to TC and AI, and may include epigenetic and gene expression changes. Methylation of gene promoters causes gene silencing and is associated with tumors, resulting in alterations of gene expression. Evaluation of expressional differences and promoter methylation may lead to the evaluation of new markers for diagnostic or prognostic use in tissue obtained from needle core biopsies.
We have evaluated a pool of six matched patient tumor and TAHN samples by microarray analysis. RNA expression between the two sample pools was found to be altered when compared to disease-free prostate gene expression. We have also characterized the methylation status of the genes GSTP1, Rar-β2, APC, and RassF1A in prostate cancer cell lines (PC-3, DU145, LnCaP and C4-2b), four disease-free prostate tissues (from autopsy material) and eight matched patient tumor and TAHN tissue samples. The cell lines demonstrated high levels of methylation, whereas the disease-free prostates demonstrated a lack of methylation for all genes. Methylation levels were more variable in the matched patient samples, and more samples are being characterized to evaluate potential patterns.
We conclude that in addition to genomic instability, alterations in mRNA expression and the methylation status of gene promoters are occurring in TAHN tissues and display potential clinical implications.

Innovative Minds in Prostate Cancer Today (IMPaCT), September 5-8, 2007, Atlanta, Georgia

Evaluation of Genomic Instability by Methylation Status in the Abnormal Prostate
Christina M Haaland; Christopher Heaphy; Kimberly Butler; Marco Bisoffi; Jeffrey Griffith

Abstract:
Prostate cancer is the second most common cause of cancer related death in men after lung cancer, with incidence of prostate cancer increasing significantly with advanced age. It is currently accepted that tumorigenesis is a multi-step process where there is accumulation of genetic and epigenetic changes that alter the normal regulatory mechanisms controlling cellular proliferation. However, not enough is yet known about the processes of tumorigenesis and disease progression, creating limitations in detection, treatment and prevention of this cancer. This study is designed to look for better methods of detection and prognostic markers regarding prostate cancer to reduce the risk of mortality associated with current treatment modalities.
In retrospective studies, our laboratory has shown that telomere content (TC), a proxy for telomere length, correlates strongly with clinical outcome in prostate and breast cancers. Additionally, studies within our lab suggest that the extent of allelic imbalance (AI), another marker of genomic instability where microsatellite repeats are measured for changes in heterozigosity, is associated with both TC and prognosis in prostate cancers. Most importantly, we have shown that the changes in TC and AI observed in tumors are also present in tumor adjacent histologically normal (TAHN) tissues. This suggests that genetic changes indicative of

tumor progression precede histological differentiation, and further implies that markers of genetic instability in seemingly normal tissue proximal to prostate tumors also have prognostic significance.
Another important question is whether the genetic changes in the TAHN prostate tissues are limited to TC and AI, or also include epigenetic changes, particularly methylation of key genes. Methylation of gene promoters causes their expression to be silenced. Detection of the methylation status is done through the use of very specific primers design for the regions where methylation occurs-CG repeats found mainly within the promoters of genes. Following treatment with sodium bisulfite, CG repeats without methylation are changed into TG repeats, allowing for this selective detection. Evaluation of promoter methylation, if differential, may allow for several new markers to be evaluated for diagnostic or prognostic use in tissue obtained from needle core biopsies. Currently we have characterized the methylation status of the genes GSTP1, Rar-beta2, APC, and RassF1A in both prostate cancer model cell lines and patient samples of both tumor and matched nearby histologically normal tissues. While there is increasing levels of methylation with increasingly aggressive tumor cell lines, the picture is more variable in the matched patient samples. However none of these genes is methylated in normal prostate tissue samples. Methylation studies are also ongoing to determine how methylation changes in tumor and nearby histologically normal tissues of the abnormal prostate, and how these changes may be relevant to detection of prostate cancer. Additionally, microarray studies are being used to help determine future investigations. These are being carried out with pooled, matched samples of tumor and nearby normal prostate tissue samples from six patients.

IMPACT statement: The results of this work will impact patient care and treatment through more sensitive detection methods and tailoring of individual patient care through outcome prediction.

Journal Articles

C.M. Heaphy, M.Bisoffi, C.A. Fordyce, **C.M. Haaland**, W.C. Hines, N.E. Joste and J.K. Griffith. Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors. International Journal of Cancer, 119:108-116, 2006. (Appendix B). Note: Ms. Haaland's participation in this project occurred prior to her receipt of the PCRP Award.

C.M. Heaphy, W.C. Hines, K.S. Butler, **C.M. Haaland**, G. Heywood, E.G. Fischer, M. Bisoffi and J.K. Griffith. Measurement of Genome-wide Allelic Imbalance in Human Tissue Using a Multiplex PCR System. Journal of Molecular Diagnotics (in press). (Appendix C).

V. Conclusions

TC and AI studies are ongoing regarding the prospective arm of specific aim one, and completed for the retrospective aspect of the same aim. Samples are currently being analyzed to determine if the changes found in the nearby histologically normal tissue may have diagnostic and/or prognostic value. Early results imply this may be the case.

Regarding specific aim 2 and the methylation studies, it has been found that there is altered methylation within the field of histologically normal tissues. Additional samples are being analyzed to verify these findings. It has also been shown that methylation of normal, disease-free tissue in not found at the gene promoters under investigation.

Microarray studies are now complete and results are being validated. Of note is the finding that Egr-1 is elevated not only in prostate tumor tissue, but also in the histologically normal tissues surrounding the tumor, indicating that Egr-1 may have potential as a biomarker. This result has been validated by RT-PCR.

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Appendix A

Figure 1

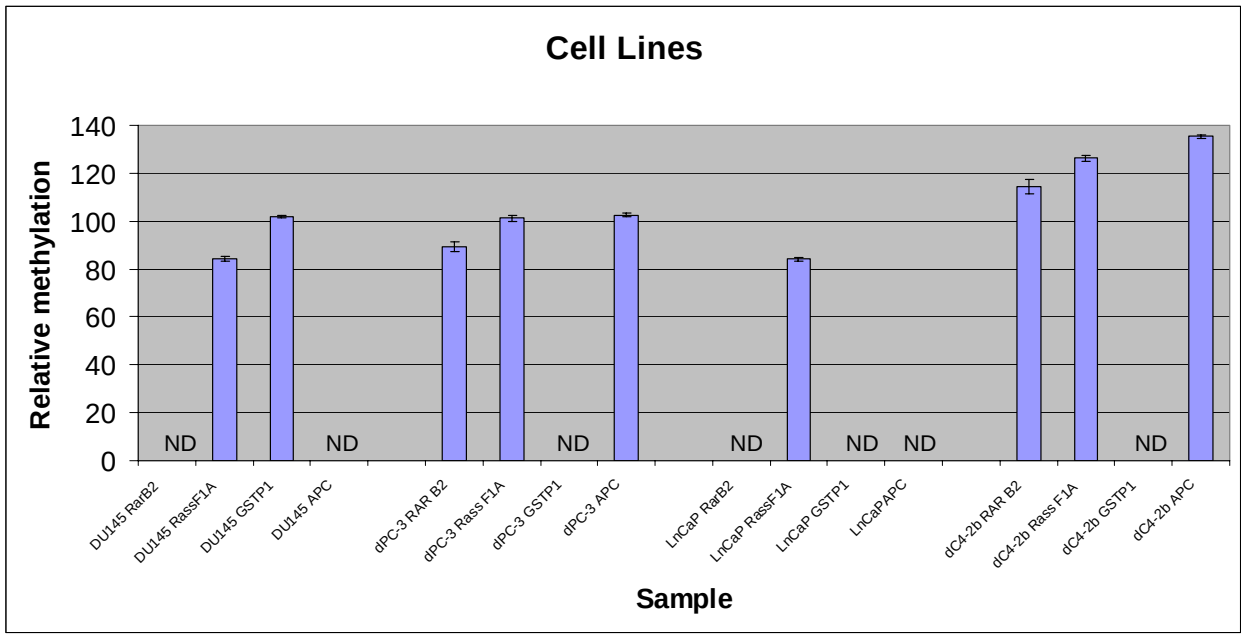

Figure 1. The methylation status of 4 prostate cancer cell line models: DU-145, derived from a brain metastasis and hormone insensitive, PC-3, an androgen independent model derived from a bone metastasis, LnCaP, lymph node derived and hormone sensitive, and C4-2b, a bone metastasis, androgen independent cell line. The promoter methylation status for the genes Rar-B2, APC, Rass F1A, and GSTP-1 have been analyzed in these cell lines. B-actin is used as an internal reference to normalize the assay (not shown). ND=none detected.

Figure 2

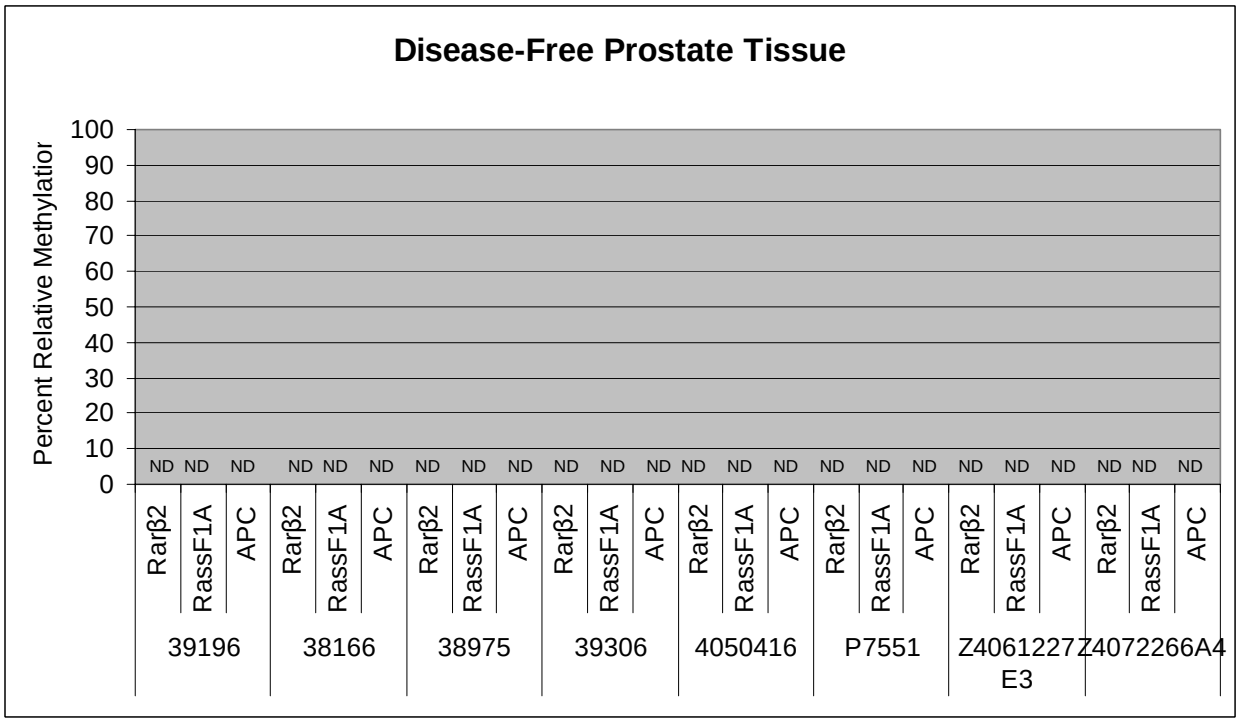

Figure 2. DNA from disease-free prostate tissues. Included are a post-mortem DNA sample of prostate tissue from an infant (P7551), and six post-mortem DNA samples from adults shown to be free of prostate disease (39306, 39196, 38166, 28975, 29206, Z4061227E3, Z4072255A4). All samples were analyzed for methylation with the promoters for Rar-B2, APC, Rass F1A, and

GSTP-1. In all cases all gene promoters were found to be unmethylated. B-actin was the internal reference control used to normalize assay results (not shown). ND=none detected.

Figure 3

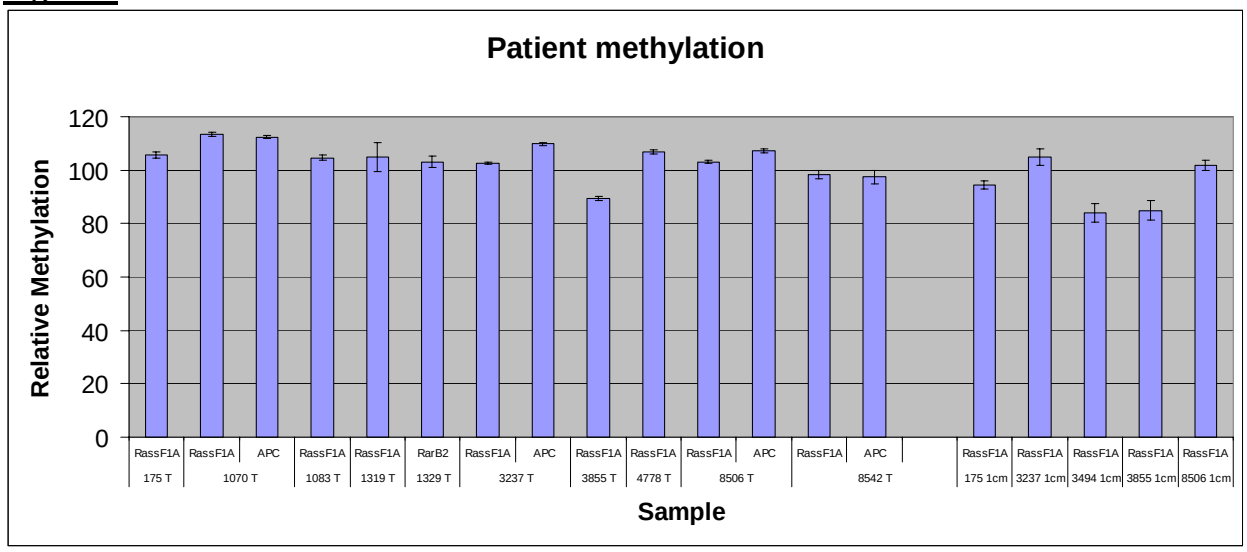

Figure 3. Graph represents all instances of promoter methylation found for RassF1A, APC, or Rar-β2 among 12 matched patient sample sets. Methylation relative to B-actin is shown on the left of the graph, sample designation is shown along the bottom. 'T' indicates tumor tissue, '1cm' indicates nearby histologically normal tissue. Tumor tissue (T) methylation levels are shown on the left, nearby histologically normal tissues (1cm) are on the right. B-actin was used as the internal control (not shown).

Figure 4

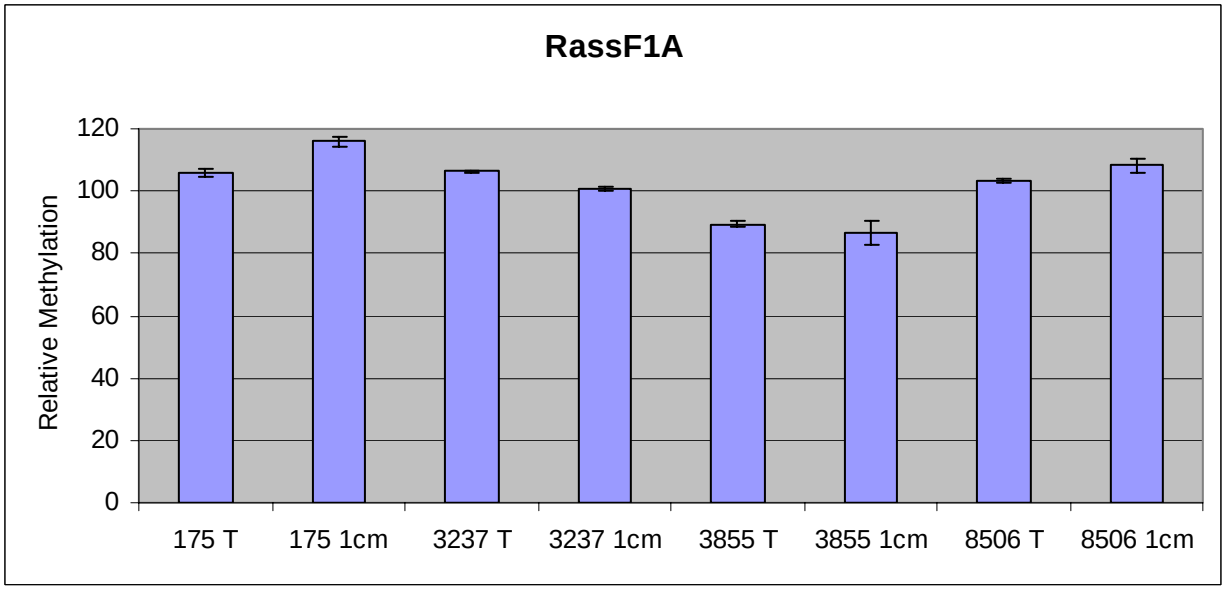

Figure 4. Promoter methylation found for the RassF1A promoter. Methylation relative to B-actin is shown on the left of the graph, sample designation is shown along the bottom. 'T' indicates tumor tissue, '1cm' indicates nearby histologically normal tissue. RassF1A was found to be methylated not only in the tumor tissue, but also the matched nearby histologically normal tissue of 4 of the patient samples examined. These findings indicate that epigenetic alterations exist beyond the tumor. B-actin was used as the internal control (not shown).

Figure 5

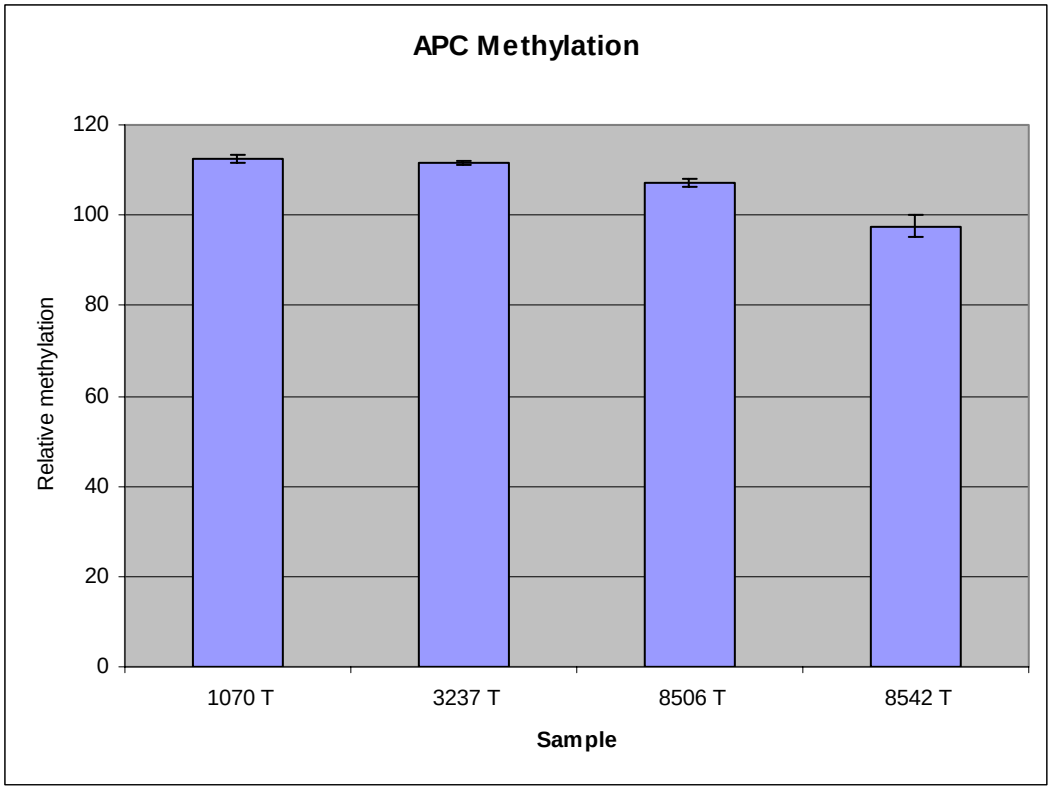

Figure 5. Promoter methylation results for APC. Methylation relative to B-actin is shown on the left of the graph, sample designation is shown along the bottom. 'T' indicates tumor tissue. The gene promoter was found to be methylated in the above tumor tissues, however not in the nearby histologically normal tissues of any patient samples examined. B-actin was used as the internal control (not shown).

Figure 6

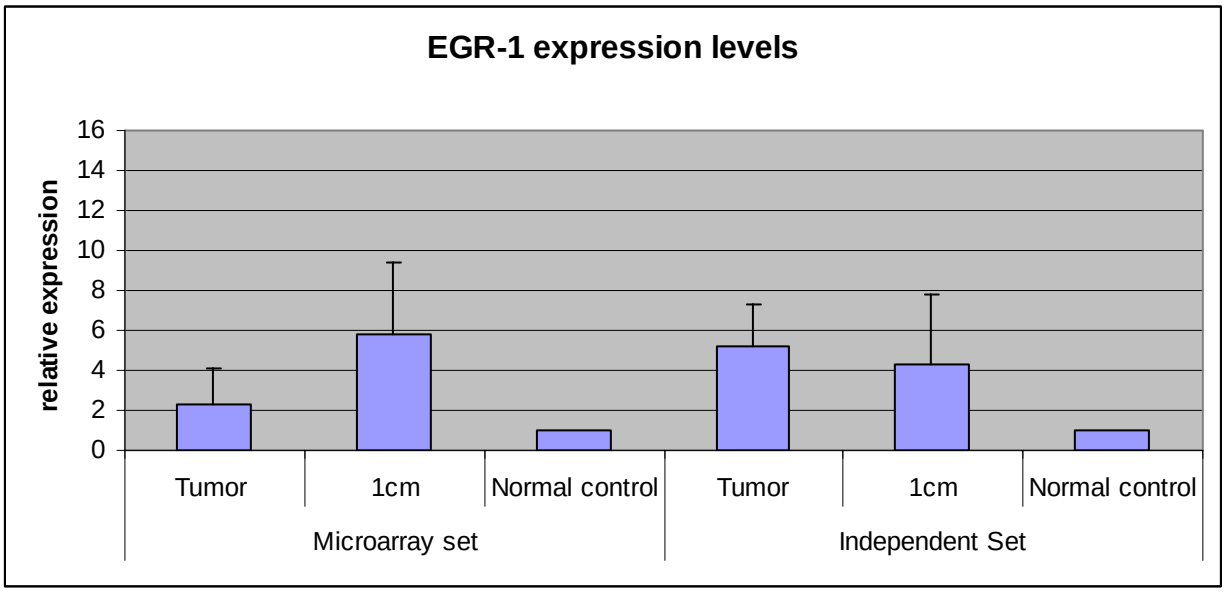

Figure 6. Results of Real-Time RT-PCR analysis of Egr-1 expression levels. Two sets of RNA were evaluated for Egr-2 expression. The RNA of the original 6 patient samples used for the microarray study was analyzed and the results are summarized for tumor, nearby histologically normal, and the pooled 'normal' RNA used for the microarray, on the left side of the graph. The right side of the graph shows the results of an independent RNA set of 6 matched patient samples. TBP was used as the normal expression control for all samples analyzed.

Figure 7

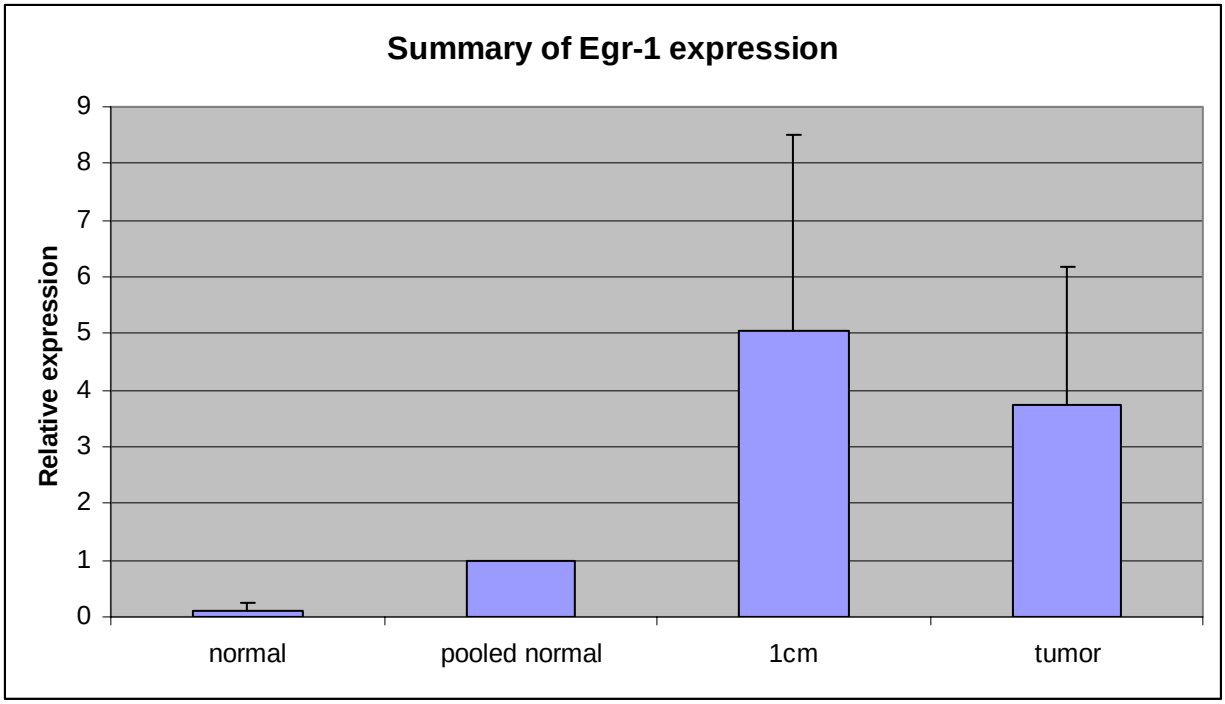

Figure 7. Summary of Egr-1 expression levels. Relative expression was determined for a total of 12 samples of tumor tissues, an additional set of 12 matched nearby histologically normal tissues, the pooled normal prostate tissue RNA used for the microarray control, and an additional 6 individual truly disease-free prostate tissues. Results indicate that Egr-1 expression is normally low in normal prostate tissues compared to tumor and nearby histologically normal tissues.

Table 1

Designation	Oligonucleotide Sequence
Rar-β2 Forward	5'-CGA GAA CGC GAG CGA TTC-3'
Rar-β2 Reverse	5'-CAA ACT TAC TCG ACC AAT CCA ACC-3'
Rar-β2 Probe	5'-6-FAM-TCG GAA CGT ATT CGG AAG GTT TTT TGT AAG TAT TT-6-TAMSp-3'
β-actin Forward	5'-TGG TGA TGG AGG AGG TTT AGT AAG-3'
β-actin Reverse	5'-ACC CAA TAA AAC CTA CTC CTC CCT TAA-3'
β-actin Probe	5'-6-FAM-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-6-TAMSp-3'
GSTP-1 Forward	5'-AGT TGC CGC GCG ATT-3'
GSTP-1 Reverse	5'-GCC CCA ATA CTA AAT CAC GAC G-3'
GSTP-1 Probe	5'-6-FAM-CGG TCG ACG TTC GGG GTG TAG CG-6-TAMSp-3'
RassF1A Forward	5'-GCG TTG AAG TCG GGG TTC-3'
RassF1A Reverse	5'-CCC GTA CTT CGC TAA CTT TAA ACG-3'
RassF1A Probe	5'-6-FAM-ACA AAC GCG AAC CGA ACG AAA CCA-6-TAMSp-3'
APC Forward	5'-GAA CCA AAA CGC TCC CCA T-3'
APC Reverse	5'-TTA TAT GTC GGT TAC GTG CGT TTA TAT-3'
APC Probe	5'-6-FAM-CCC GTC GAA AAC CCG CCG ATT A-6-TAMSp-3'

Table 1. Primers and probes used for Q-MSP. The left column indicates the primer/probe designation, the right column indicates the sequence of the oligonucleotide and fluorescent labels (for the probes).

Table 2

Tumor	
Average	Description
6.8±1.7	β-MICROSEMINOPROTEIN PRECURSOR (PROSTATE SECRETED SEMINAL PLASMA PROTEIN)
5.5±2.7	UBIQUITIN-PROTEIN LIGASE NEDD4-LIKE
5.4±2.5	SIALIDASE 1 PRECURSOR
5.3±1.2	FATTY ACID SYNTHASE
5.3±0.6	TRISTETRAPROLINE (TTP)
5.1±0.2	GLUTAMATE CARBOXYPEPTIDASE II
5.1±0.6	X BOX BINDING PROTEIN-1 (XBP-1)
5.0±0.4	β-MICROSEMINOPROTEIN PRECURSOR (PROSTATE SECRETED SEMINAL PLASMA PROTEIN)
4.8±1.0	CALRETICULIN PRECURSOR (CRP55)
4.5±1.6	CALCIUM-TRANSPORTING ATPASE TYPE 2C, MEMBER 1
4.3±0.9	TESTICAN-1 PRECURSOR (SPOCK PROTEIN)
1 cm	
Average	Description
8.9±2.1	EARLY GROWTH RESPONSE PROTEIN 1 (EGR-1)
6.8±0.9	DIFFERENTIALLY EXPRESSED IN HEMATOPOIETIC LINEAGES
6.0±0.6	DIFFERENTIALLY EXPRESSED IN HEMATOPOIETIC LINEAGES
4.4±0.2	MUCIN 4 (FRAGMENT)
4.0±0.7	OK/SW-CL.87
4.0±1.4	P40
0.3±0.03	40S RIBOSOMAL PROTEIN S26
0.3±0.01	SUPEROXIDE DISMUTASE
0.3±0.03	MATRIX GLA-PROTEIN PRECURSOR (MGP)

Table 2. Partial list of genes found to be expressed at levels 3 fold above or below the control levels in either tumor or TAHN/1cm distant from tumor tissues. Tumor and 1cm tissues are a pool of six matched patient samples represented in equal parts. Sample sets were run in triplicate.

Table 3

	1cm background	Tumor background	Cy3/Cy5 Slide 1	Cy3/Cy5 Slide 2
Mean	1.54	1.63	1.13	1.40
St Dev	0.83	0.98	0.32	0.31
CV (%)	53.86	50.12	28.40	22.29

Table 3. Dye bias control for the microarray assays. Control RNA was labeled with either Cy 3 or Cy5 and then hybridized to the slide. The data from this control indicates that the dyes bound with near equal efficiency as seen by the comparison of the means being close to 1, as compared with the sample assays that demonstrated more variation

Appendix B

Int. J. Cancer: **119,** 108–116 (2006)

Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors

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Cancer arises from an accumulation of mutations that promote the selection of cells with progressively malignant phenotypes. Previous studies have shown that genomic instability, a hallmark of cancer cells, is a driving force in this process. In the present study, two markers of genomic instability, telomere DNA content and allelic imbalance, were examined in two independent cohorts of mammary carcinomas. Altered telomeres and unbalanced allelic loci were present in both tumors and surrounding histologically normal tissues at distances at least 1 cm from the visible tumor margins. Although the extent of these genetic changes decreases as a function of the distance from the visible tumor margin, unbalanced loci are conserved between the surrounding tissues and the tumors, implying cellular clonal evolution. Our results are in agreement with the concepts of ''field cancerization'' and ''cancer field effect,'' concepts that were previously introduced to describe areas within tissues consisting of histologically normal, yet genetically aberrant, cells that represent fertile grounds for tumorigenesis. The finding that genomic instability occurs in fields of histologically normal tissues surrounding the tumor is of clinical importance, as it has implications for the definition of appropriate tumor margins and the assessment of recurrence risk factors in the context of breast-sparing surgery.

Key words: telomere loss; allelic imbalance; genomic instability; cancer field effect; breast cancer

Genomic instability is an important factor in the progression of human cancers.[1–4] One mechanism that underlies genomic instability is loss of telomere function.[5–7] Telomeres are nucleoprotein complexes located at the ends of eukaryotic chromosomes. Telomeres in human somatic cells are composed of 1,000 to 2,000 tandemly repeated copies of the hexanucleotide DNA sequence, TTAGGG.[8] Numerous telomere binding proteins are associated with these repeat regions and are important for telomere maintenance.[9,10] Telomeres stabilize chromosome ends and prevent them from being recognized by the cell as DNA double-strand breaks, thereby preventing degradation and recombination.[11] However, telomeres can be critically shortened, and thereby become dysfunctional, by several mechanisms, including incomplete replication of the lagging strand during DNA synthesis,[12] loss or alterations of telomere-binding proteins involved in telomere maintenance,[13] and oxidative stress leading to DNA damage.[14] Alternatively, telomere loss may be compensated for by recombination[15,16] or, as seen in the majority of human cancers, by the enzyme telomerase.[17,18]

Telomeres in tumors are frequently shorter than in the matched adjacent normal tissues, presumably reflecting their extensive replicative histories.[19–21] The cause-and-effect relation between dysfunctional telomeres and genomic instability implies that shortened telomeres are also associated with altered gene expression. The latter is a primary source of phenotypic variability, which in turn drives the development of cell clones displaying progressively malignant traits, such as the potential for invasion and metastasis.[22] In agreement with this sequence of events, we and others have shown that telomere length, or its surrogate, telomere DNA content (TC), predicts the course of disease in several different malignancies, including leukemias,[23] non-small cell lung cancers,[24] neuroblastomas,[25] prostatic adenocarcinomas,[26–28] and breast carcinomas.[29,30]

Recently, Meeker and colleagues observed that telomere length abnormalities are early and frequent events in the malignant transformation of several types of cancer, including breast.[27,31,32] In addition, telomere attrition and other measures of genomic instability, such as allelic imbalance (AI) and loss of heterozygosity, demonstrate that genomic instability occurs within atypical breast hyperplasias,[33–35] histologically normal tissue proximal to breast tumors,[36–42] and, in some instances, breast tissue from women with benign breast disease.[43] Loss of heterozygosity and AI have also been found in the stromal compartment of cancer-associated breast tissues.[41,44] In addition, our own recent results identified fields of telomerase-positive cells within histologically normal tissues adjacent to breast tumors that could represent areas of premalignant cell populations.[45] Similarly, we have recently reported on the occurrence of telomere attrition in histologically normal prostatic tissue proximal to prostate adenocarcinomas.[28] These data imply that there is a reservoir of genetically unstable cell clones within histologically normal breast and prostate tissues that may represent fertile ground for tumor development. The origin and extent of this reservoir are presently undefined. However, the existence of fields of genetically altered cells, appearing histologically normal and disease-free, is consistent with the hypothesis that genomic instability arises early in breast tumorigenesis.

The primary goal of the present study was to define the extent and spatial distribution of genomic instability in histologically normal tissues surrounding breast tumors. A secondary goal was to investigate the relationship between genetic alterations in tumors and matched tumor-adjacent histologically normal (TA-HN) tissues. Towards these ends, two independent, yet conceptually linked markers of genomic instability, TC and AI, were investigated in two independent cohorts of breast tumors and their matched TA-HN tissues. One cohort represented a controlled study with tumors and matched TA-HN tissues excised at sites 1 and 5 cm from the tumor margins. The second cohort consisted of archival tumor specimens and matched TA-HN tissues excised at unknown distances from the tumor margin. Our results show that breast tumors reflect the properties of the matched TA-HN breast tissues, including the conservation of unbalanced alleles. Furthermore, our results support the hypothesis that fields of histologically normal, but genetically unstable cells provide a fertile ground for tumorigenic events in breast tissues.

Materials and methods

Breast tissue samples

Four independent cohorts of human breast tissues were used in this study. The characteristics of each of these cohorts are sum-

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TABLE I – CLINICAL CHARACTERISTICS OF TUMOR COHORTS

Cohort	*N*	Age at Dx[1]			Dx[1]			Size[2]		Node[3]		TNM Stage						
		Range	Median	Mean	IDC	LC	DCIS	S	L	N	P	n/av	I	IIA	IIB	IIIA	IIIB	IV
1	12	26–61	53	49	10	1	1	n/av		2	10	2	0	3	2	2	3	0
2	38	35–75	48	50	36	2	0	4	32	7	29	2	2	5	14	11	0	2
3	48	31–89	54	56	44	4	0	8	40	19	29	0	11	13	15	8	1	0
4 (Normal)	20	15–48	30	29	n/a	n/a	n/a	n/a		n/a		n/a						

TNM, Tumor-Nodes-Distant Metastasis; n/a, not applicable; n/av, not available.
[1]Dx, Diagnosis of invasive ductal carcinoma (IDC), lobular carcinoma (LC), ductal carcinoma in situ (DCIS).–[2]S = small (≤2 cm), L = large (>2 cm).–[3]N = negative, P = positive.

marized in Table I. The first cohort consisted of 12 full mastectomy cases obtained consecutively from the University of New Mexico (UNM) Hospital Surgical Pathology Laboratory in 2003 and 2004. Approximately 500 mg of tissue was excised from the tumors and sites 1 and 5 cm from the visible tumor margins. After resection, the tissues were immediately frozen in liquid nitrogen. Sections (10–12 μm) were prepared and stained with hematoxylin and eosin by the Human Tissue Repository Service of the UNM Department of Pathology. The sections were examined microscopically to define their histological status. In addition, serial sections of the breast tumors were collected and stored at −70°C until used for isolation of genomic DNA.

The second cohort was provided by the New Mexico Tumor Registry (NMTR) and consisted of 38 archival, paraffin-embedded ductal or lobular carcinomas and matched, histologically normal breast tissues from women who had undergone radical mastectomies or lumpectomies between 1982 and 1993. The histologically normal breast tissues originated from different blocks than the tumor tissues and were obtained at the time of dissection from sites outside the visible tumor margins. Generally, the sections were selected to contain high epithelial cell fractions.

The third cohort was obtained from the University of New Mexico Solid Tumor Facility and consisted of 48 frozen archival invasive ductal or lobular carcinomas from women who had radical mastectomies or lumpectomies between 1982 and 1993. Unlike cohorts 1 and 2, matched, histologically normal breast tissues were not available for the tumors in cohort 3.

The fourth cohort was obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN) and contained 20 normal, disease-free breast tissue samples from women undergoing reduction mammoplasty (NBRST-RM). In addition, peripheral blood lymphocytes (PBLs) were obtained from 59 women previously diagnosed with breast cancer. The women ranged in age from 25 to 74 years, with a mean of 53 years. All tissues used in this study were anonymous, and experiments were performed in accordance with all federal guidelines as approved by the University of New Mexico Health Science Center Human Research Review Committee.

TC assay

Telomere length measurements can be affected by both extraneous factors, such as tissue specimens' age and means of preservation and storage, and inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To minimize the confounding effects of extraneous factors, we previously described a slot blot method for titrating the TC in fresh, frozen or paraffin-embedded tissues up to 20 years old.[46,47] TC measured by this method is directly proportional to telomere length measured by Southern blot.[47] However, in contrast to Southern blotting, the TC assay can be performed with as little as 5 ng of genomic DNA,[46] and is insensitive to fragmentation of DNA to less than 1 kb in length.[47] Thus, there is excellent agreement between TC measured in paired tissues stored either frozen, or formalin-fixed in paraffin at room temperature.[28,30] Therefore, TC is a sensitive and convenient proxy for telomere length, particularly for applications where genomic DNA is fragmented or scant, such as in sections of archival, paraffin-embedded tissues comprising the second cohort of breast tumors, which contains specimens that are over 20 years old.

TC was measured as described previously.[46] Briefly, DNA was isolated from frozen or paraffin-embedded tissues and blood samples, using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56°C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5′-TTAGGG-3′)$_4$-FAM (IDT, Coralville, IA), was hybridized to the genomic DNA, and the membranes were washed to remove nonhybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP®-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm® for 2–10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images, using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal of three replicate tumor DNAs compared to the same amount of a placental DNA standard (typically 20 ng). In addition to placental DNA, DNA purified from HeLa cells, which has approximately 30% of placental TC was frequently included to confirm the reproducibility of the assay.

AI assay

DNA (approximately 1 ng) was amplified using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA), using the manufacturer's protocol. Each multiplex PCR reaction amplifies 16 short tandem repeat (STR) microsatellite loci from independent locations in the genome (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA). Each of the PCR primers is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, allowing the simultaneous resolution of 16 amplicons of similar size. PCR products were resolved by capillary gel electrophoresis and detected using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA). The height of each fluorescence peak in the electropherograms was quantitated using the ABI Prism GeneScan and Genotype Analysis software (Applied Biosystems, Foster City, CA) and a ratio of the peak heights of each pair of heterozygous allelic amplicons was calculated. By convention, the allele with the greater fluorescence intensity was designated the numerator. Thus, the ratio was always ≥1.0, with 1.0 representing the theoretical ratio for normal alleles.

Statistical analysis

Statistical analyses were performed using the JMP® statistical package (SAS Institute, Cary, NC), choosing a significance level of 0.01. The nonparametric two-sided Wilcoxon/Kruskal–Wallis log rank test was used to determine the comparative distribution of TC and AI in the breast tumor and TA-HN tissue specimens, as well as associations between TC and AI in the paraffin-embedded breast tumor samples of cohort 2.

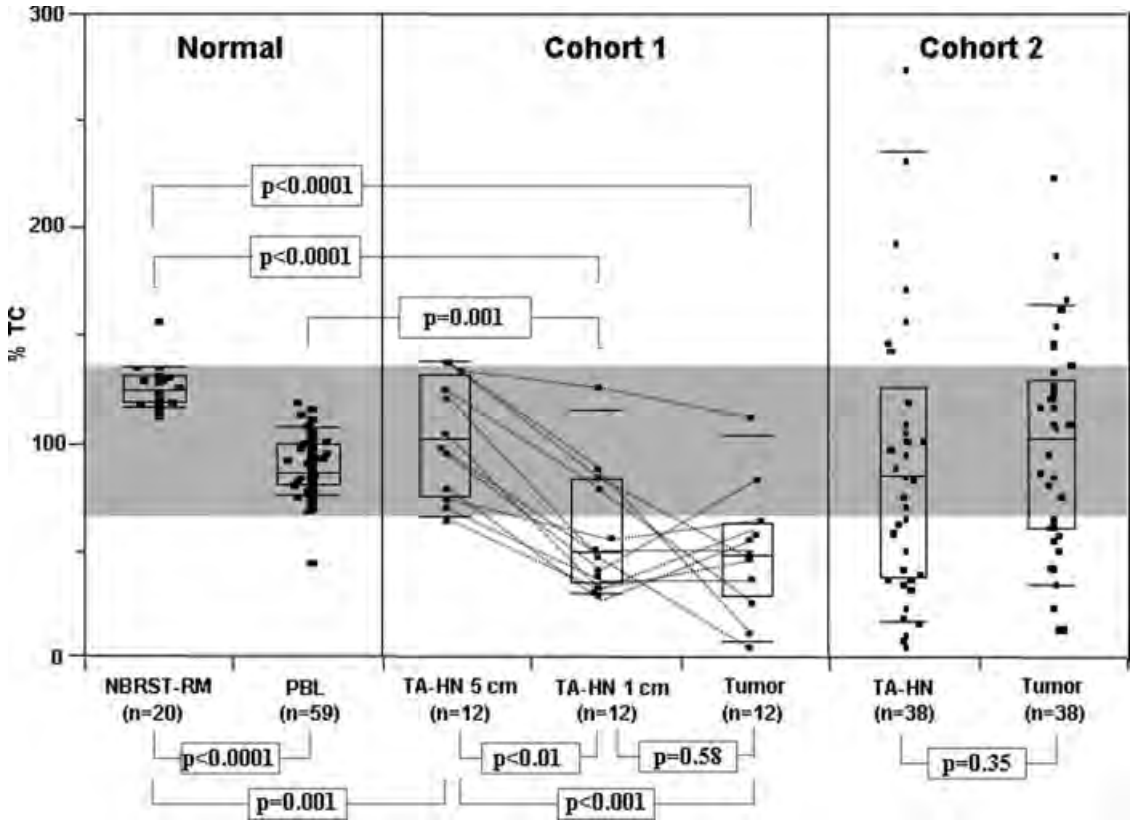

Figure 1 – Distribution of telomere DNA content (TC) in disease-free normal breast tissues from reduction mammoplasties (NBRST-RM), in peripheral blood lymphocytes (PBL), and in the breast tumor cohorts 1 and 2, including their tumor-adjacent histologically normal (TA-HN) tissues. TA-HN was excised at 1 and 5 cm from the tumor margin in cohort 1, and at unknown distances from the tumor margin in cohort 2. The number of tissues analyzed is indicated (*n*). TC is expressed as percentage of TC in placental control. The boxes represent group median (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles, respectively. In cohort 1, TC values of the individual matched samples are connected by thin lines. The gray shaded area indicates 95% of TC measurement for all normal specimens (NBRST-RM and PBLs). The *p*-values indicate comparisons between different tissue cohorts calculated by the two-sided Wilcoxon Kruskal/Wallis rank sums test. Additional statistical comparisons are mentioned in the text. *Note:* (i) Although the data points are horizontally shifted, some are still overlapping, and therefore not visible; (ii) due to the scale of the figure, two data points at values of 404% and 480% in the TA-HN set of cohort 2 are not shown.

Results

TC in normal breast tissues

To define the normal range of TC in disease-free breast tissues, the TC, a proxy for telomere length,[46,47] was measured in normal breast tissues obtained from 20 women (mean age 29) undergoing reduction mammoplasty (NBRST-RM). TC ranged from 114% to 158%, with a mean of 127% and a median of 126%, of TC in the placental DNA standard (Fig. 1). The interquartile variation (IQR), a statistical measure of the dispersion of the data, was only 12%, indicating little variation in telomere length in normal breast tissue. For comparison, TC was also measured in PBLs from 59 women (mean age 53) with a previous diagnosis of breast cancer. TC in PBLs ranged from 46% to 120%, with a mean of 90%, a median of 87% and an IQR of 19%, of the standard. The mean TC in normal breast was significantly higher than mean TC in PBLs ($p > 0.0001$). However, greater than 95% of all normal specimens (NBRST-RM and PBLs) had TC values within 70–137% of the standard. This range is interpreted to include the effects of all extraneous and inherent factors on observed TC in normal tissue, including age, tissue site, sample source and experimental variation.

Histology of cancerous and adjacent histologically normal breast tissues

The histologies of the tissues comprising two representative cases from the two independent cohorts of breast tumor tissues and matched tumor adjacent histologically normal (TA-HN) tissues are shown in Figure 2. The first cohort was composed of 12 sets of breast tumor tissues and TA-HN tissues excised 1 cm (TA-HN-1) and 5 cm (TA-HN-5) from the tumor margins. Frozen sections were stained with hematoxylin and eosin and examined microscopically. Sections of the tumors contained variable amounts of infiltrating carcinoma and ductal carcinoma *in situ* (Fig. 2*A* and 2*D*). In contrast, both TA-HN-1 and TA-HN-5 tissues had normal architecture, lobular units, ducts, and adipose tissue (Fig. 2*B*, 2*C* and 2*E*, 2*F*, respectively). Unlike the first cohort, which was composed of snap frozen tissues derived from contemporary mastectomies, the second was composed of paraffin-embedded archival tissues derived from women who had radical mastectomies or lumpectomies between 1982 and 1993. Fig. 2 shows two representative pairs of hematoxylin and eosin stained tumor (Fig. 2*G* and 2*I*) and TA-HN tissues (Fig. 2*H* and 2*J*). Infiltrating carcinoma can be seen in the tumors, while the TA-HN tissues show normal lobular architecture. Although tumor and TA-HN tissues comprising the second cohort came from different paraffin blocks, and the TA-HN tissues were obtained from sites outside the visible tumor margins, the exact distances between the sites of the TA-HN tissues and the tumors' margins are not known.

TC in tumor and adjacent histologically normal breast tissues

The spatial distribution of TC was examined in the 12 groups of breast tissues comprising the first cohort and compared with TC in the normal, disease-free breast tissues from radical mastectomy (Fig. 1). The mean TC values in the TA-HN-5 and TA-HN-1 tissues

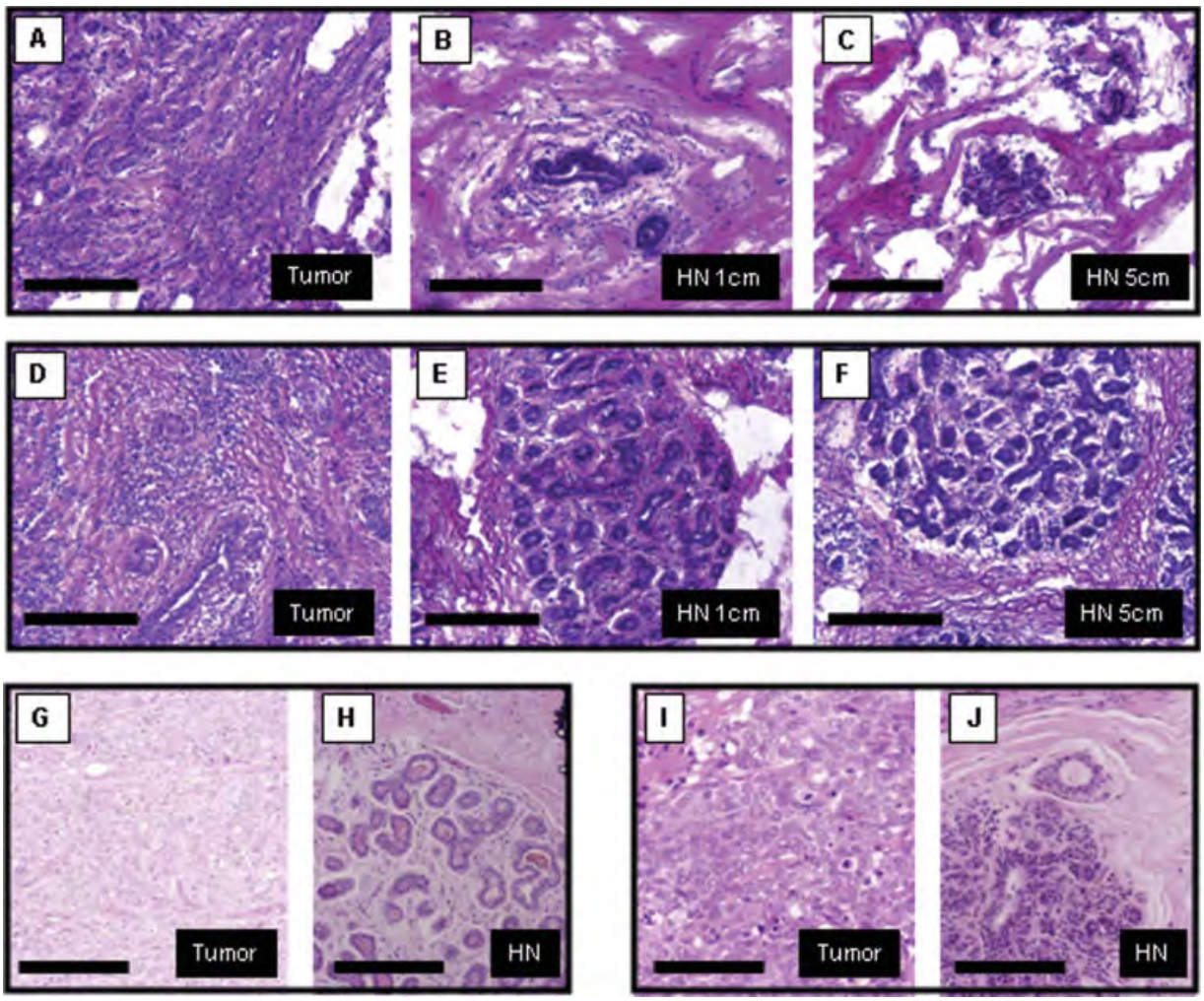

FIGURE 2 – Hematoxylin and eosin staining of human breast tissue sample sections. Two representative cases from the first (*A–F*) and second (*G–J*) cohorts are shown. Abnormal architecture with fields of infiltrating ductal carcinoma and ductal carcinoma *in situ* are seen in the tumor sections (A, D, G and I). Normal lobular and ductal architecture and adipose tissue are seen in the tumor-adjacent tissues at the indicated distance from the visible tumor margin (first cohort: B, C and E, F), or at unknown distances (second cohort: H and J). HN, histologically normal tissue; bars represent 200 μm.

were 101% and 66% of TC in the normal placental DNA standard, respectively. The mean TC value in tumors was 59%. Although the mean TC in TA-HN-5 tissues was significantly less than in NBRST-RM tissues ($p = 0.001$), it was not significantly different than the mean TC in PBLs from women of similar age ($p = 0.16$). Moreover, TC values in each of the TA-HN-5 tissues were within the range that defined >95% of all normal tissues. Since telomere length decreases with age,[48,49] it is likely that the difference between TC in the normal and TA-HN-5 tissues is due to the different ages of the two groups of women (27 *vs.* 49 years).

In contrast, mean TC in TA-HN-1 tissues was significantly less than TC in NBRST-RM tissues ($p < 0.0001$) and PBLs ($p = 0.001$), and TA-HN-5 tissues ($p < 0.01$). Mean TC in tumors also was significantly less than those in NBRST-RM tissues ($p < 0.0001$), PBLs ($p < 0.0001$) and TA-HN-5 tissues ($p < 0.001$). However, mean TC in tumor and TA-HN-1 tissues was indistinguishable ($p = 0.58$). Consistent with these findings, TC was, on average, 35% lower in each TA-HN-1 sample than in the paired TA-HN-5 sample, while the differences in TC between the TA-HN-1 and matched tumor specimens were varied, encompassing decrease, stabilization, and increase of TC with an average change of only 3% (lines in middle panel of Fig. 1). In total, TC values in 8 of 12 specimens of TA-HN-1 and 10 of 12 specimens of paired tumor tissues were outside the range that defined >95% of all normal tissues (NBRST-RM and PBLs).

Similarly, TC distribution was examined in a second, independent cohort (Fig. 1). Although the distributions of TC values in the 38 matched pairs of TA-HN and tumor tissues were broader than those measured in the first cohort (IQR = 88% and 69%, respectively), 16 of 38 TA-HN and 14 of 38 tumor specimens, respectively, had TC values less than those found in NBRST-RM tissues and PBLs, and only 9 of 38 TA-HN and 7 of 38 tumor specimens had TC values exceeding those found in all normal tissues (NBRST-RM and PBLs). A similar TC distribution was observed in a third collection of 48 frozen breast tumors (Table II), and in a collection of archival tumor and matched TA-HN prostate tissues, each collected between 1982 and 1993.[28] As observed in the comparison between tumor and TA-HN-1 specimens in the first cohort, there was no difference in mean TC in tumors and TA-HN tissues ($p = 0.35$). However, there was greater heterogeneity in the samples of the second as compared to the first cohort. Nevertheless, data from both cohorts are consistent with the conclusion that significant telomere attrition, comparable to that observed in tumors, occurs in TA-HN breast tissue. Significant telomere attrition (to a level outside the range seen in >95% of all normal tissues) occurred (i) in almost 50% (24/50) of TA-HN-1 and TA-HN

specimens, (ii) at sites at least 1 cm from the tumors' margins, and (iii) since TC is measured in bulk tissue that has not been microdissected, in a substantial fraction of the cells in the samples.

AI in tumor and adjacent histologically normal breast tissues

To investigate the extent of genomic instability in cohorts 1 and 2, tumor and TA-HN tissues were screened for AI at 16 unlinked microsatellite loci. Unlike the TC assay, which utilizes a slot blot methodology to titrate the quantity of telomere DNA in a defined amount of genomic DNA, the AI is defined by the ratio of the peak heights of allelic amplicons after PCR. Thus, it is unlikely that inherent or extrinsic factors that affect measurement of TC would similarly affect the determination of AI. To establish a baseline for the incidence of AI in normal breast tissue, 201 heterozygous loci in the 20 specimens of NBRST-RM tissues were analyzed by this approach. The mean peak height ratio was determined to be 1.18 (SD = 0.166). On the basis of these values, a highly conservative, operational definition of AI was established as a ratio of peak heights $\geq$1.68, *i.e.*, the mean + 3.0 SD. This threshold excluded more than 99% of the allelic ratios observed in the NBRST-RM tissues, and established a baseline incidence of 0.1 unbalanced loci per specimen of normal breast tissue. As shown in Figure 3, a virtually identical value, 0.08 loci per specimen, was measured in the TA-HN-5 tissues. In contrast, the mean numbers of unbalanced loci in the TA-HN-1 and tumor tissues were 0.42 and 1.25 loci per specimen, respectively, approximately 5 and 15 times higher than the incidence in the TA-HN-5 tissues. The baseline incidence of 0.1 unbalanced loci per specimen predicts that approximately 10% and 1% of normal tissues will have one and two unbalanced loci, respectively. Consistent with this prediction, 3 of 20 and 1 of 12 NBRST-RM and TA-HN-5 tissues, respectively, had one site of AI. Only one of more than 120 normal samples we have analyzed to date had 2 unbalanced loci, and none had more than 2 unbalanced loci. Accordingly, neither the NBRST-RM nor the TA-HN-5 specimens had more than one unbalanced locus. In contrast, one TA-HN-1, and 5 tumor tissues had 2 or more unbalanced loci. These data are consistent with the conclusion drawn from the TC analysis that both tumors and TA-HN-1 tissues are genetically distinct from TA-HN-5 tissue, and that both are genetically unstable.

This conclusion is further supported by results obtained with the second cohort. Microsatellite alleles were successfully amplified in 23 pairs of the 38 samples. As with the TC determinations, the distribution of the numbers of unbalanced loci was much broader in the second cohort than in the first. The mean numbers of unbalanced loci in the TA-HN tissues and matched tumors were 2.61 and 2.48 loci per specimen, respectively (Fig. 3). The mean numbers of unbalanced loci in TA-HN and tumor tissues were significantly greater than the numbers in either NBRST-RM or TA-HN-5 tissues ($p < 0.01$). The extent of AI in the tumors and their matched TA-HN tissues of the second cohort were indistinguishable ($p = 0.88$). Significantly, 74% (17/23) of TA-HN tissues and 70% (16/23) of matched tumors had 2 or more sites of AI, and 57% (13/23) and 40% (9/23), respectively, had 3 or more sites. Like the TC measurements, the independent measurement of AI, performed in two independent cohorts of paired breast tissues, indicates that at least 1 unbalanced locus is present (i) in more than 74% (26/35) of TA-HN-1 and TA-HN specimens, (ii) at sites at least 1 cm from the tumors' margins and (iii) since AI was measured in bulk tissue that was not microdissected, and the threshold for detecting AI requires that approximately 40% of the cells have lost the specific allele (see later), specific sites of AI are present in a substantial fraction of the cells.

TABLE II – TC VALUES IN NORMAL, TUMOR AND TUMOR ADJACENT, HISTOLOGICALLY NORMAL (TA-HN) TISSUES[1]

	N	Median	Mean	Min	Max	IQR
Normal tissues						
NBRST-RM	20	126	127	114	158	12
PBL	59	87	90	46	120	19
Cohort 1						
TA-HN-5	12	100	101	70	128	44
TA-HN-1	12	59	66	43	119	38
Tumor	12	57	59	24	108	27
Cohort 2						
TA-HN	38	85	106	6	480	88
Tumor	38	102	98	14	224	69
Cohort 3						
Tumor	48	105	118	65	247	60

IQR, interquartile range; NBRST-RM, normal breast tissue from reduction mammoplasty; PBL, peripheral blood lymphocytes.
[1]Data from Figure 1.

Conservation of unbalanced alleles in tumor and adjacent breast tissues

To investigate the possibility that TA-HN and tumor tissues represented early and late stages, respectively, in the clonal evolution of the cancers, we measured the frequency of conservation of unbalanced loci in the 2 cohorts of paired tumor and TA-HN tissues. As shown in Figure 4, in the first cohort, 2 of the 6 (33%) sites of AI present in TA-HN tissues were conserved in the paired tumors (left panel). Likewise, in the second cohort, 21 of the 60

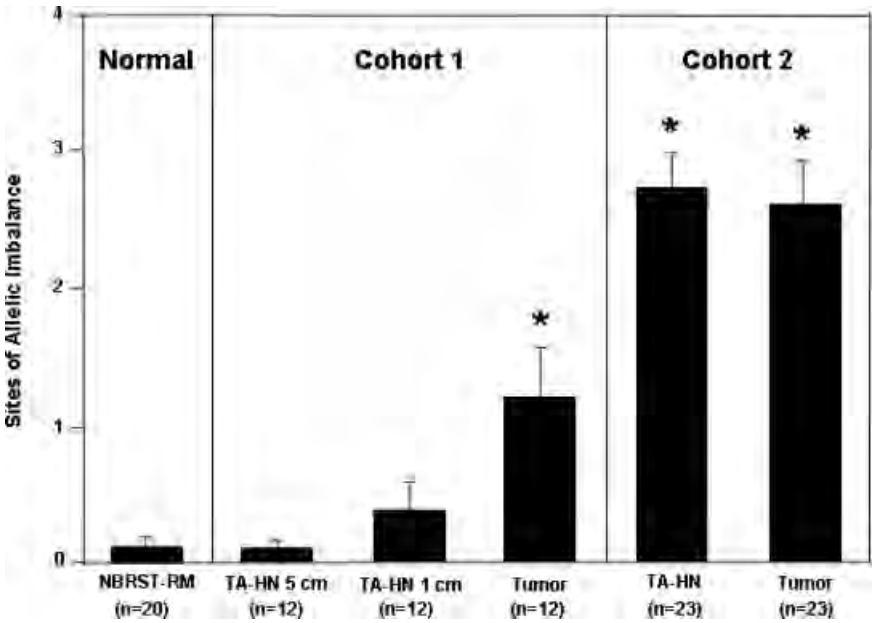

FIGURE 3 – Extent of allelic imbalance (AI) in disease-free normal breast tissues from reduction mammoplasties (NBRST-RM), and in the breast tumor cohorts 1 and 2, including their tumor-adjacent histologically normal (TA-HN) tissues. TA-HN was excised at 1 and 5 cm from tumor margin in cohort 1, and at unknown distances from the tumor margin in cohort 2. The number of tissues analyzed is indicated (*n*). The bars indicate the mean number of unbalanced loci ± standard errors. The stars indicate statistically significant differences ($p < 0.01$) from both NBRST-RM and TA-HN-5 (two-sided Wilcoxon Kruskal/Wallis rank sums test).

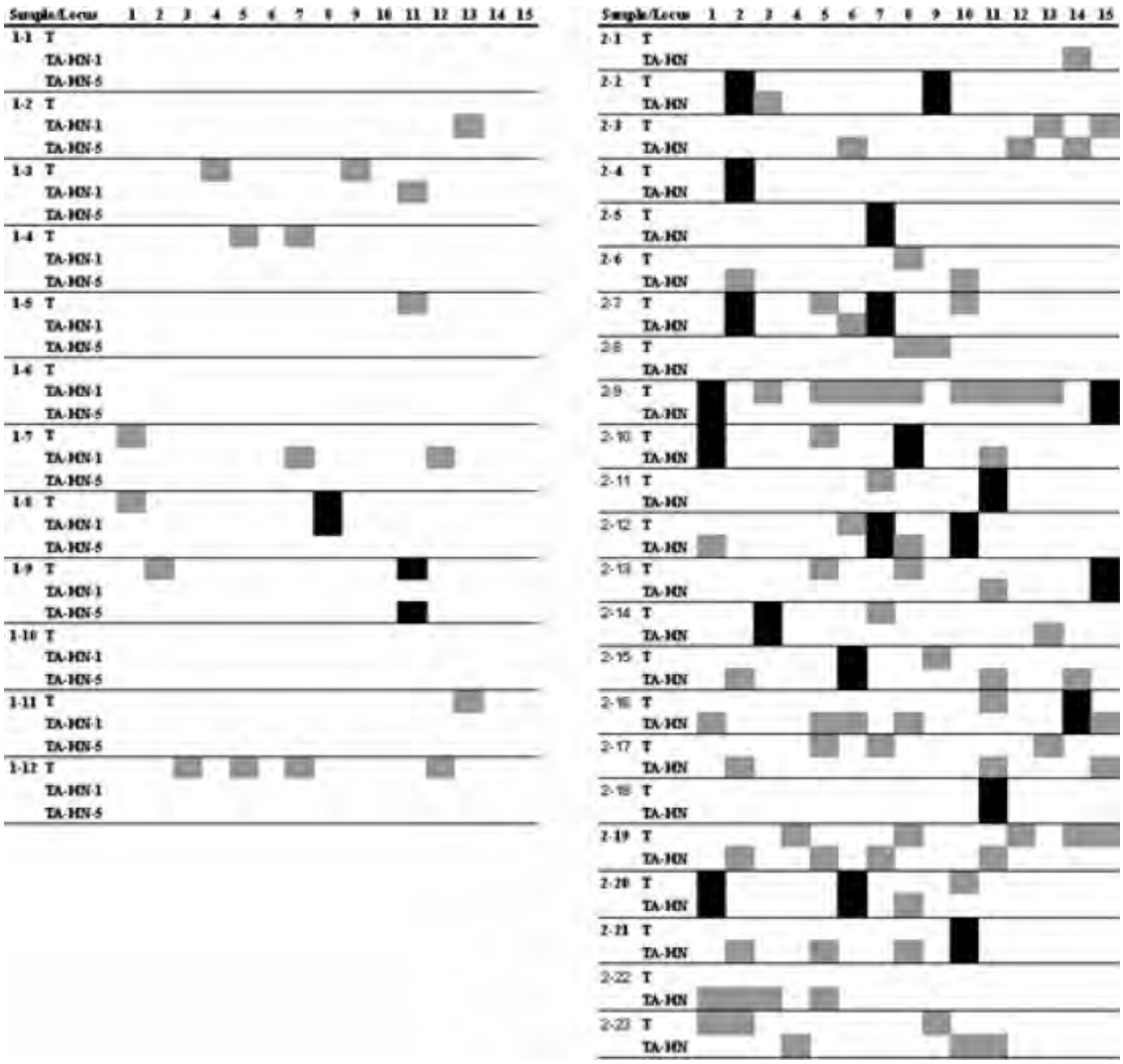

FIGURE 4 – Conservation of unbalanced alleles in matched tumor (T) and tumor-adjacent histologically normal (TA-HN) breast tissues of cohort 1 (left panel) and cohort 2 (right panel). Sites of allelic imbalances are indicated by gray boxes; sites of allelic imbalances conserved between tumor and TA-HN tissues are indicated by black boxes. The unlinked chromosomal loci are designated 1–15 and are as following (1) D8S1179, (2) D21S11, (3) D7S820, (4) CSF1PO, (5) D3S1358, (6) TH01, (7) D13S317, (8) D16S539, (9) D2S1338, (10) D19S433, (11) vWA, (12) TPOX, (13) D18S51, (14) D5S818, (15) FGA. *Note:* Homozygous amelogenin (all female samples) is not shown.

(35%) sites of AI present in TA-HN tissues were conserved in the paired tumors (right panel). The odds of this occurring by chance are estimated to be approximately 3×10^{-2} and 10^{-7} for the first and second cohorts, respectively.

Association between TC and AI in breast tumor tissues

Since telomere attrition is a source of genomic instability, and since we observed telomere attrition and increased AI in breast tumors, we determined the association between TC and AI (Fig. 5). For this analysis, microsatellite alleles were successfully amplified in 30 of the 38 breast tumor samples of cohort 2. Nonparametric 2-sided Wilcoxon/Kruskal–Wallis log rank analysis revealed a significant difference in TC in tumors with high ($\geq$3 sites) as compared to low ($\leq$2 sites) AI ($p = 0.002$).

Discussion

Although mechanistic insights into the molecular pathology of sporadic breast cancers are increasing, the question of how carcinogenesis is initiated in human breast tissues remains largely unanswered.[50–53] However, it is widely accepted that genomic instability is a prerequisite of virtually all tumors, including breast cancers, and that this instability facilitates the accumulation of further genetic alterations that result in cancer progression through clonal expansion of cells with a proliferative advantage.[1–3,51–53]

Two independent, quantitative measures of genomic instability, TC and AI, were used in this study to demonstrate that genomic instability occurs in histologically normal breast tissues adjacent to the corresponding tumors. These studies show that shortened telomeres (to a level outside the range seen in >95% of all normal tissues) and unbalanced allelic loci are present (i) in 50–75% of TA-HN and TA-HN-1 specimens, (ii) at sites at least 1 cm from the tumor margins and (iii) in a substantial fraction of the cells comprising the TA-HN tissue. This finding parallels our previous studies on tumors of the prostate and their matched TA-HN tissues,[28] and is in agreement with the work of previous investigators who reported that genetic alterations, including telomere attrition and loss of heterozygosity, occur in histologically normal tissues adjacent to breast tumors.[34–38,41–44] In these previous studies, the sites of telomere attrition, loss of heterozygosity and AI were physically distant from one another and from the tumors, albeit in most cases at undefined distances from the corresponding tumor lesions.[24,42–44] In contrast, and to our knowledge, the findings in cohort 1 represent the first

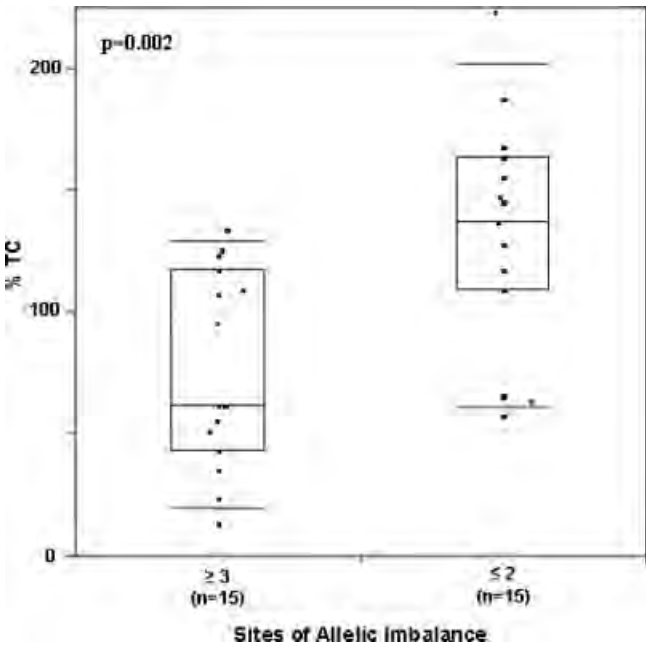

FIGURE 5 – Association between telomere DNA content and allelic imbalance in 30 breast tumor samples of cohort 2. The samples were dichotomized according to the number of genomic sites affected by allelic imbalance, i.e. ≥ 3 or ≤ 2 sites. The number of tissues analyzed is indicated (*n*). TC is expressed as percentage of TC in placental control. The boxes represent group median (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles, respectively. The nonparametric two-sided Wilcoxon/Kruskal–Wallis log rank test was used to assess the statistical significance of the difference between the means.

study in breast cancers that analyzes genomic instability at defined distances (1 and 5 cm) from the visible tumor margins. Consequently, this study reveals that genomic instability in tumor adjacent, histologically normal breast tissues is a function of distance from the tumor lesion, showing decreasing extent of genomic instability with increasing distance from the tumor margin. One explanation for these findings is that breast tumor cells exert a transforming effect on surrounding cells, leading to genetic alterations in adjacent tissues, as has been proposed for prostate cancer cells.[54,55] However, we prefer the alternate hypothesis, that breast epithelial carcinogenesis occurs at higher frequency in fields of cells with elevated genomic instability. This is supported by our observation that the occurrence of two independent markers of genomic instability, telomere attrition and unbalanced allelic loci, are highest in the tumor lesions and decrease with increasing distance from the tumor. In addition, analysis of tumors reveals an association between TC and extent of AI. Thus, we argue that telomere attrition induces genomic instability in breast tissues, and while this may not necessarily be apparent in histologically normal precancerous tissue, it is strongly displayed in tumor lesions.

Although similar conclusions can be drawn from the TC and AI analyses in each of the two cohorts, the range of TC values and the number of unbalanced loci per specimen were both greater in the second cohort. In this context, it is important to emphasize that both TC and AI reflect the average TC and peak height ratios in the cells comprising the sample; they do not provide information about the variability of TC or AI *between* individual cells. Consequently, the ability to detect specific changes in TC or AI diminishes as the number and types of cells in the sample increases. On the basis of the DNA yields, we estimate that there were approximately 20 times more cells in the samples comprising the first cohort (median $\sim 10^6$ cells), than the second cohort (median $\sim 5 \times 10^4$ cells). This difference reflects the relative amounts of tissue available from the fresh surgical specimens comprising the first cohort versus the sections of paraffin-embedded tissue blocks comprising the second cohort. This consideration is particularly significant in the case of the AI assay. On the basis of theoretical considerations and mixing experiments (data not shown), we estimate that imbalance at a specific locus must occur in ~40% of the cells in the sample to generate an allelic ratio of 1.68, the threshold for significance used in these studies. Thus, sites of AI that are not prevalent in the cell population are not detected, even if there are many such individual sites. In this context, it is not surprising that specific sites of AI are detectable in breast tumors, which evolve clonally.[51] However, it is remarkable that AI is detected in TA-HN tissue, as it not only reflects underlying genomic instability, but also requires *clonal* expansion of genetically altered, premalignant cell clones within histologically normal breast tissues. This interpretation is further corroborated by the fact that more than a third of unbalanced alleles in adjacent, histologically normal tissues are conserved in the matched tumors. The latter has important practical implications, as it indicates that it is not necessary to micro-dissect tissues, for example using laser capture microscopy, to detect genomic instability, using the assays described in the present study. In fact, these assays allow the selective detection of changes in cell clones undergoing expansion because of proliferative advantages.

Taken together, our results are in agreement with the concept of ''field cancerization,'' introduced by Slaughter and colleagues in 1953,[56] and more recently reviewed by others.[57–59] These authors developed the term to explain the multifocal and seemingly independent areas of histologically precancerous alterations occurring in oral squamous cell carcinomas.[56] Organ systems in which field cancerization has been implied include lung, colon, cervix, bladder, skin and breast.[57] The concept of field cancerization has also been used to explain the occurrence of genetic and epigenetic mosaicism in cancer precursor tissues.[60] Based on our results, we propose to extend the concept of field cancerization to genetic alterations in otherwise histologically normal breast tissues, and our study is the first to include TC.

In head and neck squamous carcinoma, field cancerization has been shown for relatively large tissue areas, *i.e.* up to 7 cm in diameter.[61] It is thus not surprising that our data show extensive field cancerization in tissues 1 cm outside breast tumor margins. In the present study, TC was also different between disease-free NBRST-RM tissues and TA-HN tissues excised at 5 cm from the tumor margin. However, TC was similar in TA-HN-5 tissues and PBLs from women of similar age. Since telomere length decreases with age,[48,49] the observed difference in TC between NBRST-RM and TA-HN-5 tissues is likely due to the age discrepancy between the two cohorts of women (27 *vs.* 49 years).

The existence of fields of genomic instability that support tumorigenic events also has important clinical implications. First, such fields could give rise to clonal selection of precursor cells that ultimately lead to the development of cancer.[62] In this context, our recent studies have identified the presence of telomerase-positive cell populations within histologically normal tissues adjacent to breast tumors that could represent fields of premalignant cells.[45] Second, the presence of such fields, even after surgical resection of primary tumors, may represent an ongoing risk factor for cancer recurrence or formation of secondary lesions, which occurs in up to 22% of women undergoing breast conservation therapies for small invasive and noninvasive breast cancers.[58,63,64] For these reasons, our study has practical implications for the assessment of appropriate tumor margins for breast cancer surgical procedures, secondary treatment options and prognosis, possibly including the risk for the development of new primary tumors in the contra-lateral breast.[65–67] Thus, our study also suggests that evaluation of surgical margins should include molecular, in addition to histological, techniques, thus warranting further investigations.

Acknowledgements

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Appendix C

Assessment of Genome-wide Allelic Imbalance in Human Tissue Using a Multiplex PCR System

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Abstract

Genomic instability can generate chromosome breakage and fusion randomly throughout the genome, frequently resulting in allelic imbalance, which is a deviation from the normal 1:1 ratio of maternal and paternal alleles. Thus, it is reasonable to speculate that tissues with more sites of allelic imbalance have a greater likelihood of having disruption of any of the numerous critical genes that cause a cancerous phenotype, and thus may have diagnostic or prognostic significance. For this reason, it is desirable to develop a robust method to provide a global assessment of genomic instability in any tissue. To address this need, we designed an economical and high-throughput method, based on the Applied Biosystems AmpFℓSTR® Identifiler multiplex PCR system, to evaluate allelic imbalance at 16 unlinked, microsatellite loci located throughout the genome. This method provides a quantitative comparison of the extent of allelic imbalance between samples that can be applied to a variety of frozen and archival tissues. The method does not require matched normal tissue, requires very little DNA (the equivalent of approximately 150 cells) and uses commercially available reagents, instrumentation and analysis software. Greater than 99% of tissue specimens with ≥ 2 unbalanced loci were cancerous.

Introduction

It is widely accepted that genomic instability- the duplication, loss or structural rearrangement of a critical gene(s) - occurs in virtually all cancers[1], and in some instances has diagnostic, prognostic or predictive significance. Thus, it is not surprising that tumor progression is reflected by allelic losses or gains in genes that regulate aspects of cell proliferation, apoptosis, angiogenesis, invasion and, ultimately, metastasis.[2,3]

There are several technologies available to detect allelic imbalance (AI), which is a deviation from the normal 1:1 ratio of maternal and paternal alleles. For example, chromosome painting techniques can identify AI in cytological preparations.[4,5] However, these methods are poorly suited for high-throughput applications and analysis is limited to a relatively small cellular field, thus increasing potential sampling error. Single nucleotide polymorphism (SNP) arrays can be used for high-resolution genome-wide genotyping and loss of heterozygousity (LOH) detection.[6-8] For example, the development of a panel of 52 microsatellite markers that detects genomic patterns of LOH [9-11] has been utilized for breast cancer diagnosis and prognosis. However, this approach requires matched referent (normal) DNA and these organ-specific panels may not be informative for other cancer types, thus limiting their applicability across multiple tumor types.

Larger panels of SNPs may be used for genome-wide analysis, for example the Affymetrix 10K and 100K SNP mapping arrays.[12-13] Likewise, Illumina BeadArrays with a SNP linkage-mapping panel,[14] allow allelic discrimination directly on short genomic

segments surrounding the SNPs of interest, thus overcoming the need for high-quality DNA.[8] Lips and colleagues have shown that Illumina BeadArrays can be used to obtain reliable genotyping and genome-wide LOH profiles from formalin-fixed, paraffin-embedded (FFPE) normal and tumor tissues.[15] Another method of detecting segmental genomic alterations is comparative genomic hybridization (CGH). CGH identifies copy-number changes by detecting DNA sequence copy variations throughout the entire genome and mapping them onto a cytogenetic map supplied by metaphase chromosomes.[16] Alternatively, array CGH maps copy number aberrations relative to the genome sequence by using arrays of BAC or cDNA clones as the hybridization target instead of the metaphase chromosomes.[17-21] However, all these approaches, while robust, require costly reagents, specialized equipment, and the sheer amount of data produced from these analyses complicate the interpretation of results.

For these reasons, and as outlined by Davies et al.,[22] it is desirable to develop a general, economical, and high-throughput method to provide a global assessment of genomic instability in any tissue, independent of the nature and composition of the specimen and the availability of matched, normal tissue. To address this need, we developed a method to measure the ratio of maternal and paternal alleles at 16 unlinked, microsatellite short tandem repeat (STR) loci in a single multiplexed PCR reaction. The assay, which is based on the Applied Biosystems AmpFℓSTR® Identifiler system, can be performed with only 1 ng of genomic DNA, uses commercially available primers and reagents, and common instrumentation and analysis software. Thus, it is an attractive alternative to current methods that is readily adaptable to most clinical laboratory environments.

Materials and Methods

Tissue Acquisition: All tissues were provided by the University of New Mexico Solid Tumor Facility, unless otherwise specified. Buccal cells were collected from oral rinses of volunteers. The Cooperative Human Tissue Network (Western Division, Nashville, TN) provided frozen normal and tumor renal tissues, obtained by radical nephrectomy, frozen normal breast tissues, obtained by reduction mammoplasty, and normal frozen prostate tissues, obtained through autopsy. A set of FFPE prostate tumors, obtained by radical prostatectomy, were provided by the Cooperative Prostate Cancer Tissue Resource (http://www.cpctr.cancer.gov). Duodenal FFPE tumor tissues were obtained from the Mayo Clinic (Rochester, MN). Pancreatic FFPE normal and tumor tissues were obtained from the Department of Pathology at the University of New Mexico. Frozen endometrial tumor tissues were obtained through the Gynecologic Oncology Group (Philadelphia, PA). All specimens lacked patient identifiers and were obtained in accordance with all federal guidelines, as approved by the UNM Human Research Review Committee.

DNA Isolation and Quantification: DNA was isolated from all tissue samples using the DNeasy® silica-based spin column extraction kit (Qiagen; Valencia, CA) and the manufacturer's suggested animal tissue protocol. FFPE samples were treated with xylene and washed with ethanol prior to DNA extraction. DNA concentrations were measured using the Picogreen® dsDNA quantitation assay (Molecular Probes, Eugene, OR) using a λ phage DNA as the standard as directed by the manufacturer's protocol.

Multiplex PCR Amplification of STR Loci: The AmpFℓSTR® Identifiler kit (Applied Biosystems, Foster City, CA) was used to amplify genomic DNA at 16 different short tandem repeat (STR) microsatellite loci (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA) in a single multiplexed PCR reaction, according to the supplier's protocol. Linear amplification of allelic PCR products is a prerequisite for ratiometric determination of AI. Therefore, each PCR reaction was limited to 28 cycles, as determined in preliminary studies. The 16 primer sets are designed and labeled with either 6-FAM, PET, VIC or NED to permit the discrimination of all amplicons in a single electrophoretic separation. The PCR products were resolved by capillary electrophoresis using an ABI Prism® 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Fluorescent peak heights were quantified using ABI Prism GeneScan® Analysis software (Applied Biosystems, Foster City, CA). Allelic ratios were calculated using the peak height, rather than the peak area, as suggested in previous studies.[23-25] For simplicity, the allele with the greater fluorescence was always made the numerator, as to always generate a ratio ≥ 1.0.

Statistical Analysis: A Pearson Chi-square test was performed using SAS JMP® software version 9.1 (SAS Institute Inc., Cary, NC) to examine the relationship between the extent of AI and tissue type, using a significance level of 0.05.

Results

The 16 allelic microsatellite loci amplified by the AmpFℓSTR® Identifiler primer sets are unlinked, and can be used to assess AI simultaneously at multiple heterozygous sites throughout the genome. This is technically possible because each amplicon is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, thus allowing the resolution of amplicons of similar size. Figure 1 shows the sizes of VIC-labeled amplicons derived from a representative specimen of matched normal and tumor renal tissue (the fluorescent channels showing the PET, 6-FAM, and NED-labeled products are not shown). Within Figure 1A, illustrating the results from the normal tissue specimen, two of the allelic pairs are homozygous (D13S317, D16S539), as indicated by a single peak, and three of the allelic pairs are heterozygous (D3S1358, TH01, D2S1338), as indicated by two peaks. Although the peak heights varied between different loci, ostensibly due to different PCR efficiencies, the peak heights of the paired alleles were similar. Theoretically, the ratio of any two heterozygous alleles following PCR amplification would be 1.0 in normal tissues. To test this premise, the ratios of paired alleles' signal intensities were compared at 320 heterozygous loci in buccal cells from 27 healthy individuals. As expected, the mean ratio was near 1.0 (mean =1.15, SD 0.18). We expect that approximately 97.5% of all allelic ratios in normal tissues would fall within 2.5 SD of the mean, and therefore operationally defined an allelic ratio of >1.60 (mean + 2.5 SD) as a site of AI. Applying this threshold to the 27 analyzed buccal samples, only 8 sites of AI were detected out of the 320 heterozygous loci, thus representing a mean of 0.30 unbalanced loci per sample. Figure 1B illustrates the results of the tumor tissue matched to the normal sample in

Figure 1A. Within this sample, two of the three heterozygous loci in the renal tumor tissue amplified by the VIC-labeled primer sets have peak height ratios of >1.60, identifying them as sites of AI.

To determine whether AI determinations were reproducible, the assay was repeated within a random subset of the buccal samples. Figure 2A shows that 193 of the 198 (97.5%) loci measured were correctly categorized upon repeating the experiment; whereas, only 5 of the 198 (2.5%) loci initially designated as sites of AI could not be confirmed. Two loci changed from sites without AI (≤ 1.60) to sites of AI (> 1.60) and three loci changed from sites of AI to sites without AI.

We next confirmed that the differences in AI detected by this approach reflected true differences in the ratio of the alleles, and not experimental artifact (e.g. differential PCR amplification efficiency), we constructed defined mixtures of DNAs from the paired normal and tumor tissue shown in Figure 1. As shown in Figure 2B for the D3S1358 locus, there was a linear relationship (R^2=0.965) between the ratio of alleles measured in the assay and the composition of the mixture. Similar results were obtained for each of the other loci exhibiting a site of AI (TH01: R^2=0.973; VWA: R^2=0.981; D18S541: R^2=0.953). In contrast, the composition of the mixture had no effect on the allelic ratios of loci not exhibiting AI (data not shown).

The operationally-defined threshold for AI was validated by measuring the allelic ratios for 1382 heterozygous loci in an independent test set comprised of 118 normal samples

consisting of bone (n=2), breast (n=10), buccal (n=53), lymph node (n=5), peripheral blood lymphocytes (PBL) (n=18), pancreas (n=6), placenta (n=3), prostate (n=4), renal (n=16) and tonsil (n=1) tissues (Figure 3A). In this sample set of normal tissues, only 32 of 1382 heterozygous loci were designated sites of AI, thus representing a mean of 0.27 unbalanced loci per sample, comparable to the 0.30 unbalanced loci per sample in the original normal sample set. In summary, 88 (74.6%), 29 (24.6%), and 1 (0.8%) of the 118 normal tissues specimens contained 0, 1 and 2 loci with AI, respectively.

We hypothesized that AI was associated with gene disruption and aberrant expression, implying that cancerous tissues would have more sites of AI than normal tissues. To test this hypothesis, we next measured the frequency of AI in 2792 heterozygous loci in a set of 239 frozen or FFPE tumor samples consisting of AML (n=8), breast (n=39), CML (n=3), duodenal (n=23), endometrial (n=78), pancreas (n=6), prostate (n=47), and renal (n=35) tissues. As shown in Figure 3B, 37 (15.5%), 41 (17.2%), and 161 (67.4%) of the 239 tumor tissues specimens contained 0, 1 and ≥ 2 loci with AI, respectively. In contrast to the normal tissues, 611 sites of AI were detected, thus representing a mean of 2.56 unbalanced loci per sample, nearly 10 times greater than the frequency in the normal tissues ($p < 0.0001$). In summary, 162 of 357 tissue specimens had ≥ 2 unbalanced loci, of which >99% were cancerous.

Discussion

Manifestations of genomic instability, such as AI, are widespread in solid tumors.[1] There have been numerous studies of these abnormalities and several techniques, including

chromosome painting, array CGH and SNP arrays, have emerged to analyze these differences between normal and tumor tissues.[4-21] However, these methods are typically costly, time intensive, and need a matched referent (normal) DNA sample for analysis. For this reason, it is desirable to develop general, economical, high-throughput methods to quantify the extent of AI in the genome of any tissue, independent of the nature and composition of the specimen and the availability of matched, normal tissue.

Using our newly developed assay and interpretation scheme to assess the extent of genome-wide unlinked AI in human tissues, we have shown in a set of 239 samples that 67% of the tumors contained two or more sites of AI, as compared to 0.8% of the normal samples, which represents an almost 84 fold difference. It must also be noted that this method provides a minimum estimate of AI, since the assay cannot discriminate between homozygous alleles and complete loss of heterozygosity in the absence of matched normal tissue. However, this limitation is mitigated by the near ubiquitous presence of normal tissue within tumors which allows for the assessment of AI in samples without requiring analysis of matched normal tissue. This is an important consideration in the potential evaluation of biopsy tissue, which may contain multiple clones of genetically altered cells superimposed on a background of normal stromal and epithelial cells and obtaining matched normal tissue may be difficult.

Altered gene expression resulting from genomic instability is a cause of cancer progression. We therefore hypothesized that cancerous tissues would have more sites of AI than normal tissues. Consistent with this hypothesis, >99% of tissues with ≥2 sites of

AI were cancerous. Therefore, we are currently investigating the possibility that the *number* of sites of AI in cancer tissue is a reflection of its stage of progression, and therefore may be correlated with clinical parameters or prognosis.

In conclusion, we describe here a simple method for assessing the extent of AI throughout the genome. This method has a number of significant advantages over existing technologies, such as chromosome painting, array CGH and SNP arrays, and as a molecular based assay may be utilized clinically in conjunction with histological techniques. The advantages of this method are that: (i) it is robust, reproducible and provides a quantitative basis for comparing the extent of AI between samples; (ii) it does not require matched normal tissue; (iii) it utilizes commercially available reagents, instrumentation and analysis software; (iv) it can be applied to a variety of fresh, frozen and archival tissues; (v) it requires very little DNA (the equivalent of approximately 150 cells); and (vi) >99% of tissues with ≥2 sites of AI were cancerous.

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Figure Legends

Figure 1. Electropherograms of VIC-labeled amplicons from a matched normal and renal carcinoma sample. PCR was performed and the resulting amplicons resolved as described in Materials and Methods. Only VIC-labeled amplicons are shown. In this particular sample, the D3S1358, THO1 and D2S1338 loci are heterozygous and D13S317 and D16S539 loci are homozygous. Fluorescent intensity is shown on the y-axis and amplicon size, in base pairs, is shown on the x-axis. The ratios of the fluorescent intensities of each allelic pair of heterozygous loci are shown. Loci with allelic ratios of >1.60 are defined as sites of allelic imbalance for matched normal (A) or tumor (B) tissue.

Figure 2. Reproducibility and effect of admixtures of matched normal and renal carcinoma DNA on allelic peak height ratios. (A) Allelic peak height ratios were determined for 198 heterozygous loci in16 normal buccal samples. The plot represents the first determination (x-axis) and the second determination (y-axis). The region defined by the gray shaded box represents all the loci that were determined not to be a site of AI on both determinations. The labeled points (allelic peak height ratios for both determinations) represent the five loci that were not correctly identified upon repeating the experiment. (B) The specified admixtures were generated using DNA from a matched pair of normal renal tissue and renal cell carcinoma as shown in Figure 1. Data from the heterozygous D3S1358 locus are shown. The allelic ratios are 1.09 in the normal renal tissue and 2.02 in the renal carcinoma. The best-fit line was generated by linear regression and has a correlation coefficient (R^2) of 0.965.

Figure 3. Frequency of allelic imbalance in normal and tumor tissues. The numbers of sites of allelic imbalance (i.e. 0, 1, ≥2) were determined in 118 samples of normal tissue (A) and in 239 samples of tumor tissue (B). The number of specimens in each tissue set (n) is indicated below the set designation. Abbreviations: Lymph Node: LN; Peripheral Blood Lymphocytes: PBL; Acute Myelogenous Leukemia: AML; Chronic Myelogenous Leukemia: CML; Endometrial: Endo. See Materials and Methods for additional details.

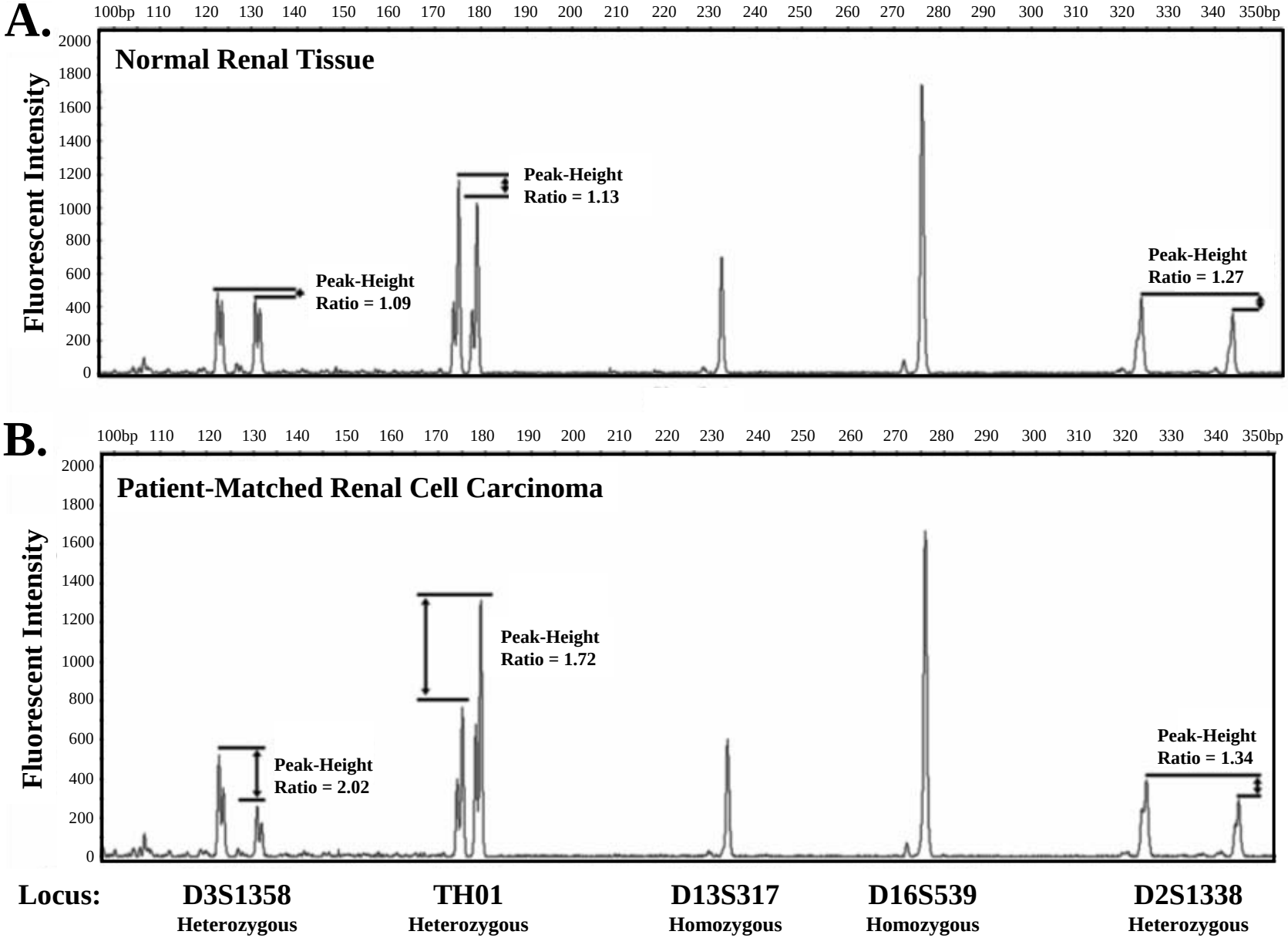
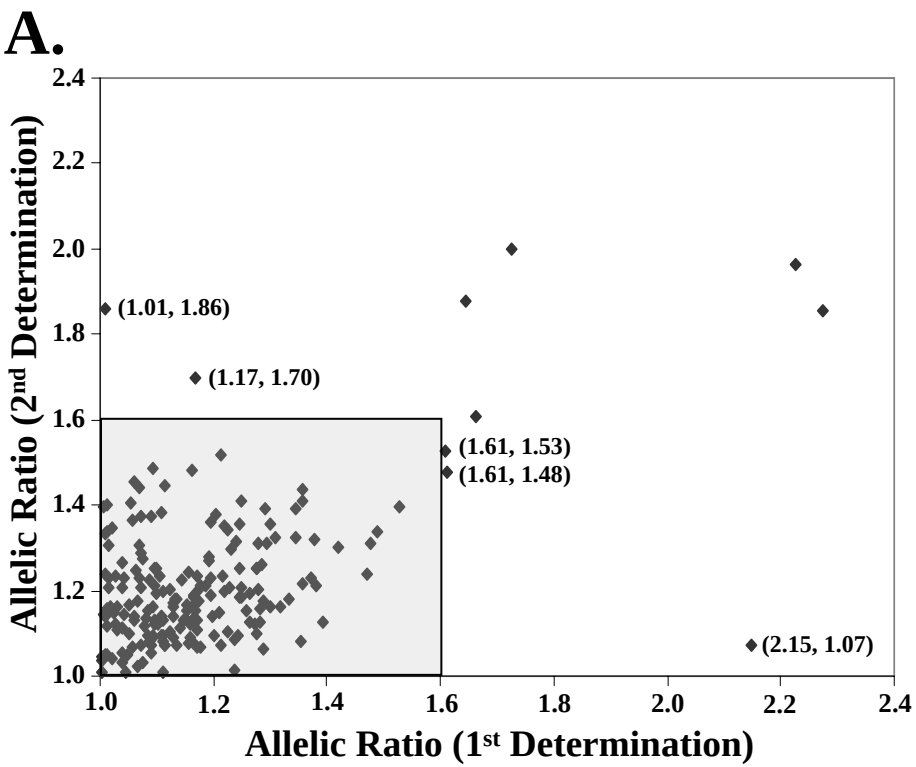

A.
Normal Renal Tissue
Fluorescent Intensity
100bp 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350bp
2000 1800 1600 1400 1200 1000 800 600 400 200 0
Peak-Height Ratio = 1.09
Peak-Height Ratio = 1.13
Peak-Height Ratio = 1.27
B.
Patient-Matched Renal Cell Carcinoma
Fluorescent Intensity
100bp 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350bp
2000 1800 1600 1400 1200 1000 800 600 400 200 0
Peak-Height Ratio = 2.02
Peak-Height Ratio = 1.72
Peak-Height Ratio = 1.34
Locus:
D3S1358 Heterozygous
TH01 Heterozygous
D13S317 Homozygous
D16S539 Homozygous
D2S1338 Heterozygous

Figure 1


A.
Allelic Ratio (2nd Determination)
Allelic Ratio (1st Determination)
(1.01, 1.86)
(1.17, 1.70)
(1.61, 1.53)
(1.61, 1.48)
(2.15, 1.07)
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4

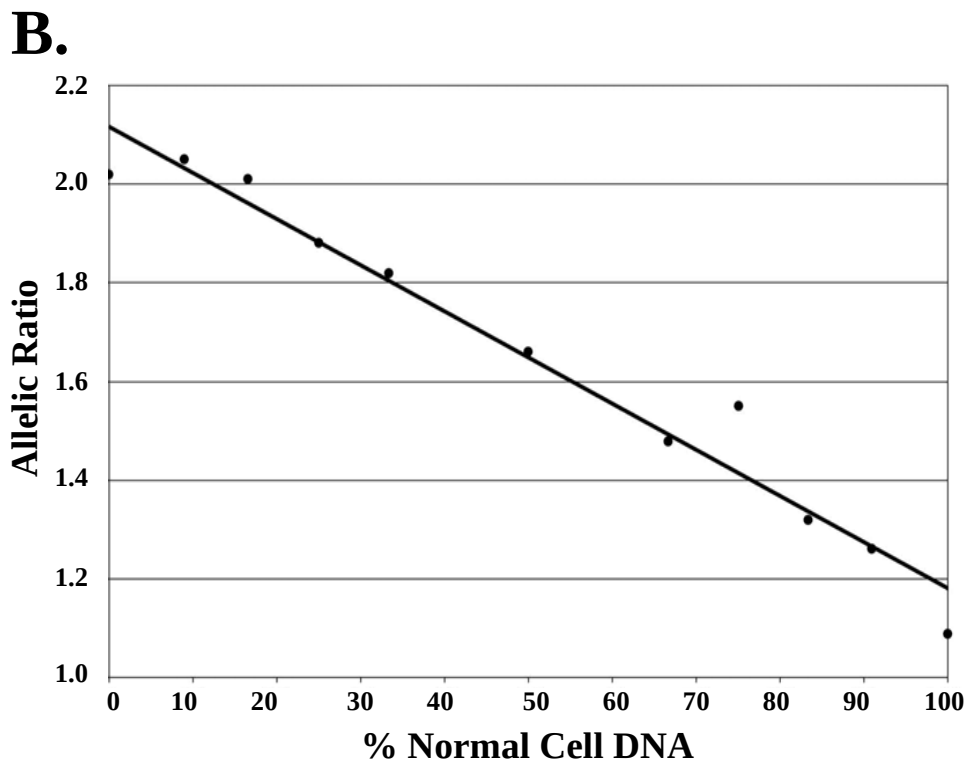

B.
Allelic Ratio
% Normal Cell DNA
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0
10
20
30
40
50
60
70
80
90
100

Figure 2

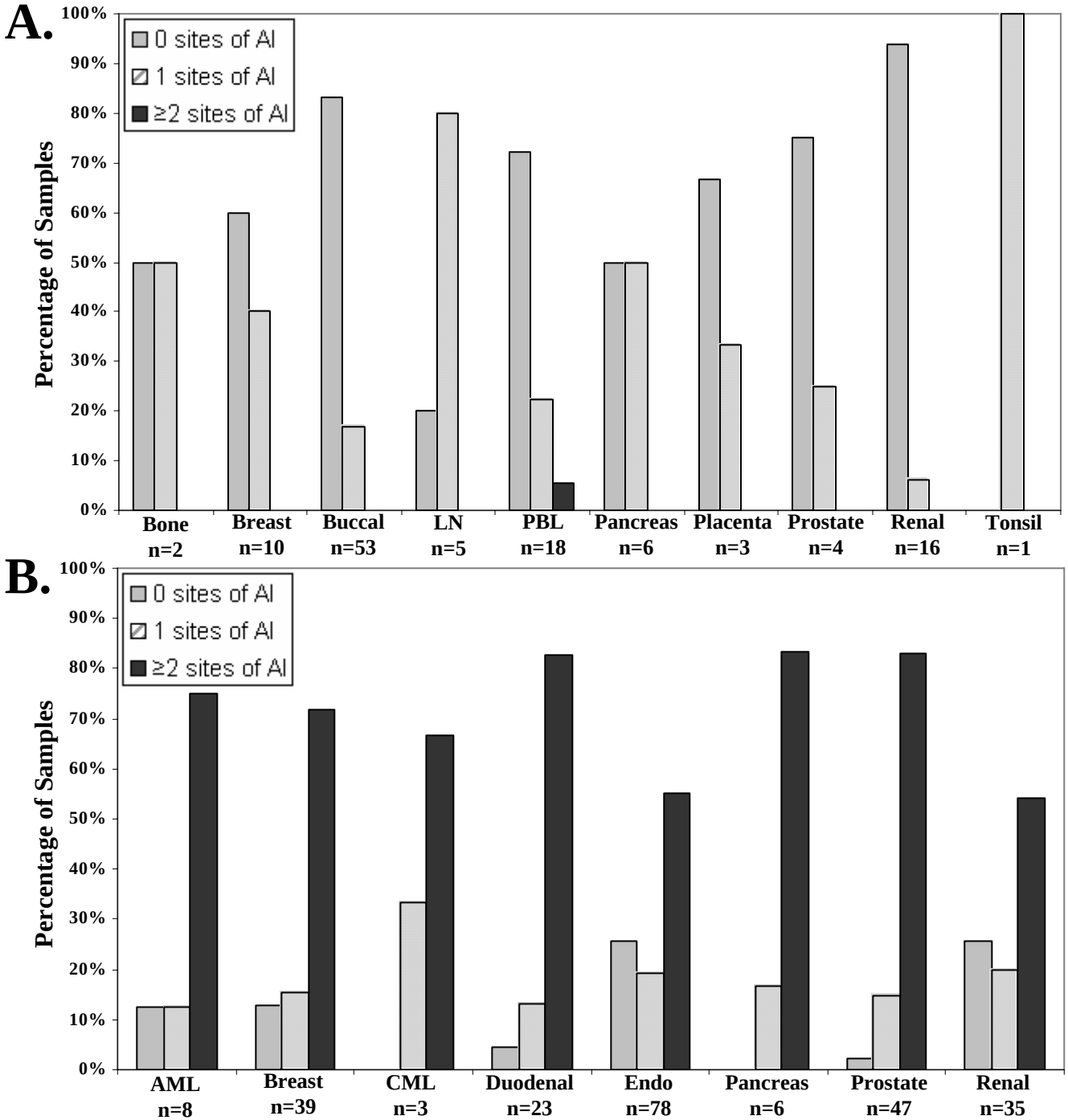

A.
0 sites of AI
1 sites of AI
≥2 sites of AI
Percentage of Samples
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Bone n=2
Breast n=10
Buccal n=53
LN n=5
PBL n=18
Pancreas n=6
Placenta n=3
Prostate n=4
Renal n=16
Tonsil n=1
B.
0 sites of AI
1 sites of AI
≥2 sites of AI
Percentage of Samples
AML n=8
Breast n=39
CML n=3
Duodenal n=23
Endo n=78
Pancreas n=6
Prostate n=47
Renal n=35

Figure 3